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(54) Title: TIE, A NOVEL ENDOTHELIAL CELL RECEPTOR TYROSINE KINASE

(57) Abstract

The cloning, sequencing and expression of a novel receptor tyrosine kinase, termed tie, is described. The tie precursor comprises 1138 amino acid residues, about 1117 residues of which comprise the mature tie. The tie extracellular domain contains distinct stretches of amino acid sequence having features of the imunoglobulin, epidermal growth factor and fibronectin type III repeat protein families. Alternative splicing creates variants of tie which lack one of the epidermal growth factor homology domains. A specific tyrosine phosphorylated 117 kD glycoprotein is detected by specific tie-antisera from cultured cells expressing the tie gene. The tie mRNA is expressed in cultured endothelial cells as well as in a few tumor cell lines. In situ hybridization of human fetal and mouse embryonic tissues shows specific expression in endothelial cells of blood vessels. The tie DNAs and polypeptides of the invention may be useful in the diagnosis and treatment of certain diseases involving endothelial cells and their tie receptor such as neoplastic diseases involving tumor angiogenesis, wound healing, thromboembolic diseases, atherosclerosis and inflammatory diseases.

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TIE, A NOVEL ENDOTHELIAL CELL RECEPTOR TYROSINE KINASE

FIELD OF THE INVENTION

The present invention relates generally to the field of genetic engineering and more particularly to genes for receptor tyrosine kinases, their insertion into recombinant DNA vectors, and the production of the resulting proteins in recipient strains of micro-organisms and recipient eukaryotic cells. More specifically, the present invention is directed to tie, a novel receptor tyrosine kinase, to nucleotide sequences encoding tie, and to methods for the generation of DNAs encoding tie and their gene products. Tie DNAs and polypeptides of the invention may be useful in the diagnosis and treatment of certain diseases involving endothelial cells and associated tie receptors, such as neoplastic diseases involving tumor angiogenesis, wound healing, thromboembolic diseases, atherosclerosis and inflammatory diseases.

BACKGROUND OF THE INVENTION

The cellular behavior responsible for the development, maintenance, and repair of differentiated cells and tissues is regulated, in large part, by intercellular signals conveyed via growth factors and similar ligands and their receptors. The receptors are located on the cell surface of responding cells and they bind peptides or polypeptides known as growth factors as well as other hormone-like ligands. The results of this interaction are rapid biochemical changes in the responding cells, as well as a rapid and a long-term readjustment of cellular gene expression. Several receptors associated with various cell surfaces may bind specific growth factors.

Tyrosine phosphorylation is one of the key modes of signal transduction across the plasma membrane. Several currently known protein tyrosine kinase genes encode transmembrane receptors for polypeptide growth factors and

hormones such as epidermal growth factor (EGF), insulin, insulin-like growth factor-I (IGF-I), platelet derived growth factors (PDGF-A and -B), and fibroblast growth factors (FGFs). Heldin *et al.*, *Cell Regulation*, *1*: 555-566 (1990); Ullrich, *et al.*, *Cell*, *61*: 243-54 (1990). Growth factor receptors of endothelial cells are of particular interest due to the possible involvement of growth factors, such as FGFs, in several important physiological and pathological processes, such as vasculogenesis, angiogenesis, atherosclerosis, and inflammatory diseases. Folkman, *et al. Science*, *235*: 442-447 (1987). Also, the receptors of several hematopoietic growth factors are tyrosine kinases; these include c-fms, which is the colony stimulating factor 1 receptor, Sherr, *et al.*, *Cell*, *41*: 665-676 (1985), and c-kit, a primitive hematopoietic growth factor receptor reported in Huang, *et al.*, *Cell*, *63*: 225-33 (1990).

On the basis of structural similarities, the receptor tyrosine kinases may be divided into evolutionary subfamilies. Ullrich, *et al. Cell*, *61*: 243-54 (1990). Such subfamilies include, EGF receptor-like kinase (subclass I) and insulin receptor-like (subclass II) kinase, each of which contains repeated homologous cysteine-rich sequences in their extracellular domains. A single cysteine-rich region is also found in the extracellular domains of the eph-like kinases. Hirai, *et al.*, *Science*, *238*: 1717-1720 (1987); Lindberg, *et al. Mol. Cell. Biol.*, *10*: 6316-24 (1990); Lhotak, *et al.*, *Mol. Cell. Biol.*, *11*: 2496-2502 (1991). PDGF receptors as well as c-fms and c-kit receptor tyrosine kinases may be grouped into subclass III; while the FGF receptors form subclass IV. Typical for the members of both of these subclasses are extracellular folding units stabilized by intrachain disulfide bonds. These so-called immunoglobulin (Ig)-like folds are found in the proteins of the immunoglobulin superfamily which contains a wide variety of other cell surface receptors having either cell-bound or soluble ligands. Williams, *et al.*, *Ann. Rev. Immunol.*, *6*: 381-405 (1988).

Receptor tyrosine kinases differ in their specificity and affinity. In general, receptor tyrosine kinases are glycoproteins, which consist of (1) an extracellular domain capable of binding the specific growth factor(s); (2) a transmembrane domain which usually is an alpha-helical portion of the protein; (3) a juxtamembrane domain where the receptor may be regulated by, *e.g.*, protein phosphorylation; (4) a tyrosine kinase domain which is the enzymatic component of the receptor; and (5) a carboxyterminal tail which in many receptors is involved in recognition and binding of the substrates for the tyrosine kinase.

Processes such as alternative splicing and alternative polyadenylation have recently been reported to be capable of producing several distinct polypeptides from the same gene. These polypeptides may or may not contain the various domains listed above. As a consequence, some extracellular domains may be expressed as separate, secreted proteins and some forms of the receptors may lack the tyrosine kinase domain and contain only the extracellular domain inserted in the plasma membrane via the transmembrane domain plus a short carboxyl terminal tail.

The present invention provides a novel endothelial cell receptor tyrosine kinase which was originally identified as an unknown tyrosine kinase-homologous PCR-cDNA fragment from human leukemia cells by Partanen, *et al.*, *Proc. Natl. Acad. Sci. USA*, 87: 8913-8917 (1990). This gene and its encoded protein are called tie which is an abbreviation for the "tyrosine kinase containing immunoglobulin- and EGF-like repeats".

SUMMARY OF THE INVENTION

It is an object of the present invention to provide a DNA or RNA segment of defined structure encoding the tie receptor tyrosine kinase. A DNA or RNA according to the invention may be produced synthetically or isolated from natural sources and may be used in the production of desired recombinant DNA vectors or may be used to recover related genes from other

sources. It is a further object of the present invention to provide a recombinant DNA vector containing a heterologous segment encoding the tie receptor tyrosine kinase or a related protein which is capable of being inserted into a microorganism or eukaryotic cell for expression of the encoded protein. The present invention also provides eukaryotic cells capable of producing useful quantities of the tie receptor tyrosine kinase and proteins of similar function from multiple species. In another aspect of the invention, peptides which may be produced synthetically in a laboratory or by a microorganism which mimic the activity of the natural tie receptor tyrosine kinase protein and which may be used to produce the tie receptor tyrosine kinase or a portion thereof in eukaryotic cells in a reproducible and standardized manner are disclosed. Particularly preferred are peptides selected from the group consisting of:

(a) a first sequence:

```
1    MetValTrpArgValProProPheLeuLeuProIleLeuPheLeuAlaSerHisValGly
21   AlaAlaValAspLeuThrLeuLeuAlaAsnLeuArgLeuThrAspProGlnArgPhePhe
41   LeuThrCysValSerGlyGluAlaGlyAlaGlyArgGlySerAspAlaTrpGlyProPro
61   LeuLeuLeuGluLysAspAspArgIleValArgThrProProGlyProProLeuArgLeu
81   AlaArgAsnGlySerHisGlnValThrLeuArgGlyPheSerLysProSerAspLeuVal
101  GlyValPheSerCysValGlyGlyAlaGlyAlaArgArgThrArgValIleTyrValHis
121  AsnSerProGlyAlaHisLeuLeuProAspLysValThrHisThrValAsnLysGlyAsp
141  ThrAlaValLeuSerAlaArgValHisLysGluLysGlnThrAspValIleTrpLysSer
161  AsnGlySerTyrPheTyrThrLeuAspTrpHisGluAlaGlnAspGlyArgPheLeuLeu
181  GlnLeuProAsnValGlnProProSerSerGlyIleTyrSerAlaThrTyrLeuGluAla
201  SerProLeuGlySerAlaPhePheArgLeuIleValArgGlyCysGlyAlaGlyArgTrp
221  GlyProGlyCysThrLysGluCysProGlyCysLeuHisGlyGlyValCysHisAspHis
241  AspGlyGluCysValCysProProGlyPheThrGlyThrArgCysGluGlnAlaCysArg
261  GluGlyArgPheGlyGlnSerCysGlnGluGlnCysProGlyIleSerGlyCysArgGly
281  LeuThrPheCysLeuProAspProTyrGlyCysSerCysGlySerGlyTrpArgGlySer
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301 GlnCysGlnGluAlaCysAlaProGlyHisPheGlyAlaAspCysArgLeuGlnCysGln

321 CysGlnAsnGlyGlyThrCysAspArgPheSerGlyCysValCysProSerGlyTrpHis

341 GlyValHisCysGluLysSerAspArgIleProGlnIleLeuAsnMetAlaSerGluLeu

361 GluPheAsnLeuGluThrMetProArgIleAsnCysAlaAlaAlaGlyAsnProPhePro

381 ValArgGlySerIleGluLeuArgLysProAspGlyThrValLeuLeuSerThrLysAla

401 IleValGluProGluLysThrThrAlaGluPheGluValProArgLeuValLeuAlaAsp

421 SerGlyPheTrpGluCysArgValSerThrSerGlyGlyGlnAspSerArgArgPheLys

441 ValAsnValLysValProProValProLeuAlaAlaProArgLeuLeuThrLysGlnSer

461 ArgGlnLeuValValSerProLeuValSerPheSerGlyAspGlyProIleSerThrVal

481 ArgLeuHisTyrArgProGlnAspSerThrMetAspTrpSerThrIleValValAspPro

501 SerGluAsnValThrLeuMetAsnLeuArgProLysThrGlyTyrSerValArgValGln

521 LeuSerArgProGlyGluGlyGlyGluGlyAlaTrpGlyProProThrLeuMetThrThr

541 AspCysProGluProLeuLeuGlnProTrpLeuGluGlyTrpHisValGluGlyThrAsp

561 ArgLeuArgValSerTrpSerLeuProLeuValProGlyProLeuValGlyAspGlyPhe

581 LeuLeuArgLeuTrpAspGlyThrArgGlyGlnGluArgArgGluAsnValSerSerPro

601 GlnAlaArgThrAlaLeuLeuThrGlyLeuThrProGlyThrHisTyrGlnLeuAspVal

621 GlnLeuTyrHisCysThrLeuLeuGlyProAlaSerProProAlaHisValLeuLeuPro

641 ProSerGlyProProAlaProArgHisLeuHisAlaGlnAlaLeuSerAspSerGluIle

661 GlnLeuThrTrpLysHisProGluAlaLeuProGlyProIleSerLysTyrValValGlu

681 ValGlnValAlaGlyGlyAlaGlyAspProLeuTrpIleAspValAspArgProGluGlu

701 ThrSerThrIleIleArgGlyLeuAsnAlaSerThrArgTyrLeuPheArgMetArgAla

721 SerIleGlnGlyLeuGlyAspTrpSerAsnThrValGluGluSerThrLeuGlyAsnGly

741 LeuGlnAlaGluGlyProValGlnGluSerArgAlaAlaGluGluGlyLeuAspGlnGln

761 LeuIleLeuAlaValValGlySerValSerAlaThrCysLeuThrIleLeuAlaAlaLeu

781 LeuThrLeuValCysIleArgArgSerCysLeuHisArgArgArgThrPheThrTyrGln

801 SerGlySerGlyGluGluThrIleLeuGlnPheSerSerGlyThrLeuThrLeuThrArg

821 ArgProLysLeuGlnProGluProLeuSerTyrProValLeuGluTrpGluAspIleThr

841 PheGluAspLeuIleGlyGluGlyAsnPheGlyGlnValIleArgAlaMetIleLysLys

861 AspGlyLeuLysMetAsnAlaAlaIleLysMetLeuLysGluTyrAlaSerGluAsnAsp

881 HisArgAspPheAlaGlyGluLeuGluValLeuCysLysLeuGlyHisHisProAsnIle

901 IleAsnLeuLeuGlyAlaCysLysAsnArgGlyTyrLeuTyrIleAlaIleGluTyrAla

921 ProTyrGlyAsnLeuLeuAspPheLeuArgLysSerArgValLeuGluThrAspProAla

941 PheAlaArgGluHisGlyThrAlaSerThrLeuSerSerArgGlnLeuLeuArgPheAla

961 SerAspAlaAlaAsnGlyMetGlnTyrLeuSerGluLysGlnPheIleHisArgAspLeu

981 AlaAlaArgAsnValLeuValGlyGluAsnLeuAlaSerLysIleAlaAspPheGlyLeu

1001 SerArgGlyGluGluValTyrValLysLysThrMetGlyArgLeuProValArgTrpMet

1021 AlaIleGluSerLeuAsnTyrSerValTyrThrThrLysSerAspValTrpSerPheGly

1041 ValLeuLeuTrpGluIleValSerLeuGlyGlyThrProTyrCysGlyMetThrCysAla

1061 GluLeuTyrGluLysLeuProGlnAlaAspArgMetGluGlnProArgAsnCysAspAsp

1081 GluValTyrGluLeuMetArgGlnCysTrpArgAspArgProTyrGluArgProProPhe

1101 AlaGlnIleAlaLeuGlnLeuGlyArgMetLeuGluAlaArgLysAlaTyrValAsnMet

1121 SerLeuPheGluAsnPheThrTyrAlaGlyIleAspAlaThrAlaGluGluAla; (SEQ ID NO 1) and

(b) a second sequence in which amino acids 214 to 257 of the first formula are absent in the second sequence.

DNA and RNA molecules, recombinant DNA vectors, and modified microorganisms or eukaryotic cells comprising a nucleotide which encodes any of the peptides indicated above are also contemplated in the present invention. In particular, sequences comprising all or part of the following two DNA sequences, their complements, or corresponding RNA sequences are preferred:

1 cgctcgtcct ggctggcctg ggtcggcctc tggagtatgg tctggcgggt

51 gccccctttc ttgctcccca tcctcttctt ggcttctcat gtgggcgcgg

101 cggtggacct gacgctgctg gccaacctgc ggctcacgga cccccagcgc

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151  ttcttcctga cttgcgtgtc tggggaggcc ggggcgggga ggggctcgga
201  cgcctggggc ccgcccctgc tgctggagaa ggacgaccgt atcgtgcgca
251  ccccgcccgg gccacccctg cgcctggcgc gcaacggttc gcaccaggtc
301  acgcttcgcg gcttctccaa gccctcggac ctcgtgggcg tcttctcctg
351  cgtgggcggt gctggggcgc ggcgcacgcg cgtcatctac gtgcacaaca
401  gccctggagc ccacctgctt ccagacaagg tcacacacac tgtgaacaaa
451  ggtgacaccg ctgtactttc tgcacgtgtg cacaaggaga agcagacaga
501  cgtgatctgg aagagcaacg gatcctactt ctacaccctg gactggcatg
551  aagcccagga tgggcggttc ctgctgcagc tcccaaatgt gcagccacca
601  tcgagcggca tctacagtgc cacttacctg gaagccagcc ccctgggcag
651  cgccttcttt cggctcatcg tgcggggttg tggggctggg cgctgggggc
701  caggctgtac caaggagtgc ccaggttgcc tacatggagg tgtctgccac
751  gaccatgacg gcgaatgtgt atgcccccct ggcttcactg gcacccgctg
801  tgaacaggcc tgcagagagg gccgttttgg gcagagctgc caggagcagt
851  gcccaggcat atcaggctgc cggggcctca ccttctgcct cccagacccc
901  tatggctgct cttgtggatc tggctggaga ggaagccagt gccaagaagc
951  ttgtgccccc ggtcattttg gggctgattg ccgactccag tgccagtgtc
1001 agaatggtgg cacttgtgac cggttcagtg gttgtgtctg cccctctggg
1051 tggcatggag tgcactgtga gaagtcagac cggatccccc agatcctcaa
1101 catggcctca gaactggagt tcaacttaga gacgatgccc cggatcaact
1151 gtgcagctgc agggaacccc ttccccgtgc ggggcagcat agagctacgc
1201 aagccagacg gcactgtgct cctgtccacc aaggccattg tggagccaga
1251 gaagaccaca gctgagttcg aggtgccccg cttggttctt gcggacagtg
1301 ggttctggga gtgccgtgtg tccacatctg gcggccaaga cagccggcgc
1351 ttcaaggtca atgtgaaagt gccccccgtg cccctggctg cacctcggct
1401 cctgaccaag cagagccgcc agcttgtggt ctccccgctg gtctcgttct
1451 ctggggatgg acccatctcc actgtccgcc tgcactaccg gccccaggac
1501 agtaccatgg actggtcgac cattgtggtg gaccccagtg agaacgtgac
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1551 gttaatgaac ctgaggccaa agacaggata cagtgttcgt gtgcagctga
1601 gccggccagg ggaaggagga gaggggcct ggggcctcc caccctcatg
1651 accacagact gtcctgagcc tttgttgcag ccgtggttgg agggctggca
1701 tgtggaaggc actgaccggc tgcgagtgag ctggtccttg cccttggtgc
1751 ccgggccact ggtgggcgac ggtttcctgc tgcgcctgtg ggacgggaca
1801 cgggggcagg agcggcggga gaacgtctca tccccccagg cccgcactgc
1851 cctcctgacg ggactcacgc ctggcaccca ctaccagctg gatgtgcagc
1901 tctaccactg caccctcctg ggcccggcct cgcccctgc acacgtgctt
1951 ctgcccccca gtgggcctcc agccccccga cacctccacg cccaggccct
2001 ctcagactcc gagatccagc tgacatggaa gcacccggag gctctgcctg
2051 ggccaatatc caagtacgtt gtggaggtgc aggtggctgg gggtgcagga
2101 gacccactgt ggatagacgt ggacaggcct gaggagacaa gcaccatcat
2151 ccgtggcctc aacgccagca cgcgctacct cttccgcatg cgggccagca
2201 ttcaggggct cggggactgg agcaacacag tagaagagtc caccctgggc
2251 aacgggctgc aggctgaggg cccagtccaa gagagccggg cagctgaaga
2301 gggcctggat cagcagctga tcctggcggt ggtgggctcc gtgtctgcca
2351 cctgcctcac catcctggcc gccctttaa ccctggtgtg catccgcaga
2401 agctgcctgc atcggagacg caccttcacc taccagtcag gctcgggcga
2451 ggagaccatc ctgcagttca gctcagggac cttgacactt acccggcggc
2501 caaaactgca gcccgagccc ctgagctacc cagtgctaga gtgggaggac
2551 atcacctttg aggacctcat cggggagggg aacttcggcc aggtcatccg
2601 ggccatgatc aagaaggacg ggctgaagat gaacgcagcc atcaaaatgc
2651 tgaaagagta tgcctctgaa aatgaccatc gtgactttgc gggagaactg
2701 gaagttctgt gcaaattggg gcatcacccc aacatcatca acctcctggg
2751 ggcctgtaag aaccgaggtt acttgtatat cgctattgaa tatgcccct
2801 acgggaacct gctagatttt ctgcggaaaa gccgggtcct agagactgac
2851 ccagcttttg ctcgagagca tgggacagcc tctaccctta gctcccggca
2901 gctgctgcgt ttcgccagtg atgcggccaa tggcatgcag tacctgagtg

2951 agaagcagtt catccacagg gacctggctg cccggaatgt gctgytcgga
3001 gagaacctag cctccaagat tgcagacttc ggcctttctc ggggagaggg
3051 ggtttatgtg aagaagacga tggggcgtct ccctgtgcgc tggatggcca
3101 ttgagtccct gaactacagt gtctatacca ccaagagtga tgtctggtcc
3151 tttggagtcc ttctttggga gatagtgagc cttggaggta caccctactg
3201 tggcatgacc tgtgccgagc tctatgaaaa gctgccccag gctgaccgca
3251 tggagcagcc tcgaaactgt gacgatgaag tgtacgagct gatgcgtcag
3301 tgctggcggg accgtcccta tgagcgaccc ccctttgccc agattgcgct
3351 acagctaggc cgcatgctgg aagccaggaa ggcctatgtg aacatgtcgc
3401 tgtttgagaa cttcacttac gcgggcattg atgccacagc tgaggaggcc
3451 tgagctgcca tccagccaga acgtggctct gctggccgga gcaaactctg
3501 ctgtctaacc tgtgaccagt ctgaccctta cagcctctga cttaagctgc
3551 ctcaaggaat ttttttaact taagggagaa aaaaagggat ctggggatgg
3601 ggtgggctta ggggaactgg gttcccatgc tttgtaggtg tctcatagct
3651 atcctgggca tccttctttc tagttcagct gccccacagg tgtgtttccc
3701 atcccactgc tcccccaaca caaaccccca ctccagctcc ttcgcttaag
3751 ccagcactca caccactaac atgccctgtt cagctactcc cactcccggc
3801 ctgtcattca gaaaaaaata aatgttctaa taagctccaa aaaaa (SEQ ID NO. 2); or

a second sequence, wherein the nucleotides corresponding to positions 676 to 807 in the first sequence are absent from the second sequence.

DNA and RNA molecules containing segments of the larger sequence are also provided for use in carrying out preferred aspects of the invention relating to the production of such peptides by the techniques of genetic engineering and the production of oligonucleotide probes. Since the DNA sequence encoding the tie protein has been fully identified, it is possible to produce an entire gene by, for example, polymerase chain reaction or by synthetic chemistry using commercially available equipment, after which the gene can be inserted into

any of the many available DNA vectors using known techniques of recombinant DNA technology. Furthermore, automated equipment is also available which makes direct synthesis of any of the peptides disclosed herein readily available. Thus, the present invention may be carried out using reagents, plasmids, and microorganism which are readily available to the skilled artisan.

BRIEF DESCRIPTION OF THE DRAWINGS

The accompanying Figures 1 to 13 are provided to illustrate the invention but are not considered to be limiting thereof unless so specified. Figure 1. Nucleic and deduced amino acid sequence of the tie cDNA. The 3845 bp nucleotide sequence compiled from two overlapping cDNA clones isolated from HEL library contains an open reading frame of 1138 amino acids (marked in the single-letter code). The tie precursor begins from nucleotide number 37 and the mature tie protein from amino acid 22 (nucleotide number 100). The hydrophobic signal sequence and the putative transmembrane domain are underlined (thick lines) as are the sites for potential N-linked glycosylation (thin lines). The mature tie protein Cysteine residues found in the extracellular domain have been boxed, the tyrosine kinase domain is shown by horizontal arrows and the kinase insert with italics. The three cysteine-rich segments having homology to EGF-like domains are also boxed (EGFH I-III). Their alignment is shown in Fig. 2. The first of the EGF repeats missing in clone 3a is indicated by vertical arrows. The sequence has been deposited to GenBank/EMBL (Accession no. X60957). A is alanine, C is cysteine, D is aspartate, E is glutamate, F is phenylalanine, G is glycine, H is histidine, I is isoleucine, K is lysine, L is leucine, M is methionine, N is asparagine, P is proline, Q is glutamine, R is arginine, S is serine, T is threonine, V is valine, W is tryptophan, and Y is tyrosine.

Figure 2. A. Alignment of the EGF-like domains of tie. Comparison is made with human EGF sequence (amino acid residues 1-44) and homologous sequences in the growth factor

CRIPTO (67-108), laminin A chain (1092-1138), Drosophila melanogaster Notch (897-945) and Caenorhabditis elegans Lin-12 (204-246) developmental control proteins, human blood coagulation factor IXa (83-130) and mouse urokinase type plasminogen activator (18-65). The asterisks point out conserved residues and the homologous cysteine residues are boxed. The consensus residues for s-hydroxylation present in the repeats of Notch and factor IXa are printed in boldface. B. Comparison of the three fibronectin type III repeats of the tie protein and the first three FNIII repeats of the human LAR receptor phosphotyrosine phosphatase. The cysteine residues as well as some other consensus residues typical for immunoglobulin domains are shown above the second FNIII repeat of the tie protein.

Figure 3. Expression of tie cDNA in COS cells. COS cells were transfected with SV40-based expression vectors for tie (SV14-1, SV14-2) and FGFR-4 (C, Partanen, J., T. P. Makela, E. Eerola, J. Korhonen, H. Hirvonen, L. Claesson-Welsh, and K. Alitalo, *EMBO J. 10*: 1347-1354, 1991), labelled with ^{35}S-methionine, lysed and immunoprecipitated as described in materials and methods of example 3. Autoradiograms of the SDS-PAGE analysis of the precipitated proteins are shown. A. Identification of tie polypeptides expressed in the COS cells. HI, immune serum against s-gal-tie fusion protein; HO, preimmune serum. The immune serum was blocked with the antigen where indicated (+). B. Effect of tunicamycin on the molecular weight of the tie protein. MI, immune serum against a carboxyl terminal tie peptide; MO preimmune serum. Where indicated (+), the transfected cell cultures were labelled in the presence of tunicamycin. Mobilities of the molecular weight markers are shown on the left.

Figure 4. Immunoblot analysis of cell lines expressing the tie protein. Cell lysates of NIH3T3 cells transfected (LTR14-2) or not transfected (NEO1) with a tie expression vector as well as porcine aortic endothelial cells (PAE) were analyzed by immunoblotting with antiserum against a carboxyterminal tie peptide. The samples in the two right

most lanes (aPY, IP) were immunoprecipitated with anti-phosphotyrosine antibodies prior to immunoblotting.

Figure 5. Chromosomal mapping of the tie locus. Radiolabeled JTK14 DNA was hybridized to normal human male peripheral lymphocyte metaphase preparations; slides were washed, developed after exposure and chromosomes were G-banded to distinguish individual chromosomes. Grain localization is illustrated on the schematic chromosome 1 where each dot represents 3 grains. Some nonspecific background signal was detected on the other chromosomes; 12,6% (40/317) on other chromosomes of group A, 8,5% (27/317) on chromosomes of group B, 29.6% (94/317) on C-group chromosomes and 14.8% (47/317) on the other chromosome groups.

Figure 6. tie mRNA expression in leukemia cell lines. Poly (A)+ RNA from the indicated cell lines was analyzed by Northern blotting and hybridization with the tie cDNA probe. Hybridization with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was used as an internal control for the loading of even amounts of RNA to the analysis.

Figure 7. tie mRNA expression in endothelial cell lines. Northern blot analysis of tie mRNA expression in PAE and EA hy926 endothelial cell lines. A lane containing poly(A)+ RNA from Dami cells was included as a positive control.

Figure 8. Location of tie mRNA in endothelium of the kidney vessels by *in situ* hybridization. The dark field image showing the hybridization signal is on the top (A). A corresponding phase-contrast micrograph is shown below (B).

Figure 9. Comparison of the structure of the tie protein with some other receptor tyrosine kinases containing immunoglobulin and fibronectin type III repeats.

The open circles represent immunoglobulin loops, the open boxes fibronectin type III repeats and the filled ovals EGF homology domains. The shaded box represents the cysteine rich region of the eph-like kinases. The cytoplasmic tyrosine kinase domains are drawn as black boxes.

Figure 10. Schematic structure of the human tie receptor tyrosine kinase and comparison of its deduced amino acid sequence with two mouse tie cDNA clones (1C1D and D10E5).

The tie receptor consists of two immunoglobulin-like loops (Ig), three (or two) epidermal growth factor domains (EGF) followed by three fibronectin III like domains, a transmembrane region (TM) and two cytoplasmic tyrosine kinase domains (TK1 and TK2). Amino acid homology between mouse and human tie amino acid sequences is 96% and 95% for the segments 1C1D and D10E5, respectively. Amino acid residue symbols are as in Fig. 1.

Figure 11. Expression of tie mRNA in human tissues. Total RNA isolated from 17-19 week fetal tissues was analyzed by Northern blotting (A). Hybridization of polyadenylated RNA from human adult tissues is shown in B. The s-actin and GAPDH probes were used as internal controls for the amount of RNA loaded.

Figure 12. In situ hybridization analysis of tie mRNA expression in 12 day p.c. mouse embryo.

Shown are light-field (A) and dark-field (B,C) photomicrographs of a sagittal section hybridized with 1C1D antisense (A,B) and sense probes (C). Expression of tie mRNA is restricted to the endothelium of blood vessels. Used abbreviations: br (brain), mg (meninges), lg (lung), mb (mandible), ht (heart), vn (ventricle), at (atrium), sc (spinal cord), pv (prevertebra), and cv (posterior cardinal vein).

Figure 13. Comparison of tie mRNA (A) and factor VIII (B) expression in a 8 day p.c. mouse placenta.

Factor VIII is seen as the dark deposit surrounding the blood lacunae in (A) and the tie signal in a similar but separate section (B) is seen as white grains. As can be seen from the figure, both signals are localized to endothelial cells of blood lacunae which form the labyrinth.

DETAILED DESCRIPTION

In the description which follows, a number of terms used in recombinant DNA (rDNA) technology are extensively utilized. In order to provide a clear and consistent understanding of the specification and claims, including the scope to be given to such terms, the following definitions are provided.

Gene: A DNA sequence containing a template for an RNA polymerase. The RNA transcribed from a gene may or may not code for a protein. RNA that codes for a protein is termed messenger RNA (mRNA) and, in eukaryotes, is transcribed by RNA polymerase II. However, it is also known to construct a gene containing an RNA polymerase II template wherein a RNA sequence is transcribed which has a sequence complementary to that of a specific mRNA but is not normally translated. Such a gene construct is herein termed an "antisense RNA gene" and such a RNA transcript is termed an "antisense RNA." Antisense RNAs are not normally translatable due to the presence of translational stop codons in the antisense RNA sequence. A "complementary DNA" or "cDNA" gene includes recombinant genes synthesized by reverse transcription of mRNA lacking intervening sequences (introns).

Cloning vehicle: A plasmid or phage DNA or other DNA sequence which is able to replicate autonomously in a host cell, and which is characterized by one or a small number of endonuclease recognition sites at which such DNA sequences may be cut in a determinable fashion without loss of an essential biological function of the vehicle, and into which DNA may be spliced in order to bring about its replication and cloning. The cloning vehicle may further contain a marker suitable for use in the identification of cells transformed with the cloning vehicle. Markers, for example, are tetracycline resistance or ampicillin resistance. The word "vector" is sometimes used for "cloning vehicle."

Expression vector: A vehicle or vector similar to a cloning vehicle but which is capable of expressing a gene which has been cloned into it, after transformation into a host. The cloned gene is usually placed under the control of

(i.e., operably linked to) certain control sequences such as promoter sequences. Expression control sequences will vary depending on whether the vector is designed to express the operably linked gene in a prokaryotic or eukaryotic host and may additionally contain transcriptional elements such as enhancer elements, termination sequences, tissue-specificity elements, and/or translational initiation and termination sites. The present invention pertains both to expression of recombinant tie protein, and to the functional derivatives of this protein.

Functional Derivative: A "functional derivative" of tie protein is a protein which possesses a biological activity (either functional or structural) that is substantially similar to a biological activity of non-recombinant tie protein. A functional derivative of tie protein may or may not contain post-translational modifications such as covalently linked carbohydrate, depending on the necessity of such modifications for the performance of a specific function. The term "functional derivative" is intended to include the "fragments," "variants," "analogues," or "chemical derivatives" of a molecule. As used herein, a molecule is said to be a "chemical derivative" of another molecule when it contains additional chemical moieties not normally a part of the molecule. Such moieties may improve the molecule's solubility, absorption, biological half life, etc. The moieties may alternatively decrease the toxicity of the molecule, eliminate or attenuate any undesirable side effect of the molecule, etc. Moieties capable of mediating such effects are disclosed in Remington's Pharmaceutical Sciences (1980). Procedure for coupling such moieties to a molecule are well known in the art.

Fragment: A "fragment" of a molecule such as tie protein is meant to refer to any variant of the molecule, such as the peptide core, or a variant of the peptide core. Variant. A "variant" of a molecule such as tie protein is meant to refer to a molecule substantially similar in structure and biological activity to either the entire molecule, or to a

fragment thereof. Thus, provided that two molecules possess a similar activity, they are considered variants as that term is used herein even if the composition or secondary, tertiary, or quaternary structure of one of the molecules is not identical to that found in the other, or if the sequence of amino acid residues is not identical.

Analog: An "analog" of tie protein or genetic sequences is meant to refer to a protein or genetic sequence substantially similar in function to the tie protein or genetic sequence herein.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is directed to "tie", a novel receptor tyrosine kinase, tie-encoding nucleic acid molecules (*e.g.*, cDNAs, genomic DNAs, RNAs, anti-sense RNAs, etc.), production of tie peptides or tie protein from a tie gene and its product, recombinant tie expression vectors, tie analogs and derivatives, and diagnostic and/or therapeutic uses of tie and related proteins, tie -encoding nucleic acid molecules, tie ligands, tie antagonists and anti-tie antibodies.

EXAMPLE 1

Production of Recombinant Tie

Biologically-active tie may be produced by the cloning and expression of a tie-encoding nucleotide or its functional equivalent in a suitable host cell. Production of tie using recombinant DNA technology may be divided into a step-wise process for the purpose of description, which process includes: (1) isolating or generating the coding sequence (gene) for the desired tie; (2) constructing an expression vector capable of directing the synthesis of the desired tie; (3) transfecting or transforming appropriate host cells capable of replicating and expressing the tie gene and/or processing the gene product to produce the desired tie; and (4) identifying and purifying the desired tie product.

A. isolation of the tie gene

The nucleotide coding sequence of tie, or functional equivalents thereof, may be used to construct recombinant expression vectors which direct the expression of the desired tie product. The nucleotide coding sequence for tie is depicted in SEQ ID NO. 1. The nucleotide sequence depicted therein, or fragments or functional equivalents thereof, may be used to generate the recombinant molecules which direct the expression of the recombinant tie product in appropriate host cells. Tie-encoding nucleotide sequences may be obtained from a variety of cell sources which produce products with tie-like activities and/or which express tie-encoding mRNA. The Applicants have identified a number of suitable human cell sources for tie including endothelial cells, leukemia cells, and rhabdomyosarcoma and fibrosarcoma cells.

The tie coding sequence may be obtained by cDNA cloning from RNA isolated and purified from such cell sources or by genomic cloning. For example, the tie sequence may be amplified by polymerase chain reaction from cDNA or genomic DNA material using techniques well-known in the art. Either cDNA or genomic libraries of clones may be prepared using techniques well-known in the art and may be screened for particular tie DNAs with nucleotide probes which are substantially complementary to any portion of the tie gene. Full length clones, *i.e.*, those containing the entire coding region of the desired tie gene, may be selected for use in constructing expression vectors. Alteratively, tie-encoding DNAs may be synthesized, in whole or in part, by chemical synthesis using standard techniques.

Due to the inherent degeneracy of nucleotide coding sequences, other DNA sequences which encode substantially the same or a functionally equivalent amino acid sequence may be used in the practice of the method of the invention. Such alterations of tie nucleotide sequences include deletions, additions, or substitutions of different nucleotides resulting in a sequence that encodes the same or a functionally equivalent gene product. The gene product may contain deletions, additions, or substitutions of amino acid residues

within the sequence which result in "silent" changes thus producing a bioactive tie product. Such amino acid deletions, additions, or substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity and/or the amphipathic nature of the amino acids involved. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids indude lysine and arginine; amino acids with uncharged polar head groups or nonpolar head groups having similar hydrophilicity values indude the following: leucine, isoleucine, valine; glycine, alanine; asparagine, glutamine; serine, threonine; phenylalanine, tyrosine.

B. construction of tie expression vectors

Using the above information, a variety of recombinant DNA vectors capable of expressing the tie receptor tyrosine kinase in reasonable quantities are provided. Additional recombinant DNA vectors of related structure which code for synthetic proteins having the key structural features identified herein as well as for proteins of the same family from other sources may be produced from the tie receptor tyrosine kinase cDNA using standard techniques of recombinant DNA technology. A transformant expressing the tie receptor tyrosine kinase has been produced as an example of this technology (see EXAMPLES 3 and 4). The newly discovered sequence and structure information may be used, through transfection of eukaryotic cells, to prepare the tie receptor tyrosine kinase and its various domains for biological purposes.

C. Identification of Transfectants or Transformants Expressing tie Gene Products

The host cells which contain recombinant coding sequences and which express the biologically active, mature product may be identified by at least four general approaches: (a) DNA-DNA, DNA-RNA or RNA-antisense RNA hybridization; (b) the presence or absence of "marker" gene functions; (c) assessing the level of transcription as measured by the expression of

tie mRNA transcripts in the host cell; and (d) detection of the mature gene product as measured by immunoassay and, ultimately, by its biological activities.

In the first approach, the presence of tie coding sequences inserted into expression vectors may be detected by DNA-DNA hybridization using probes comprising nucleotide sequences that are homologous to the tie coding sequence.

In the second approach, the recombinant expression vector/host system may be identified and selected based upon the presence or absence of certain "marker" gene functions (*e.g.*, thymidine kinase activity, resistance to antibiotics, resistance to methotrexate, transformation phenotype, occlusion body formation in baculovirus, etc.). For example, if the tie coding sequence is inserted within a marker gene sequence of the vector, recombinants containing that coding sequence may be identified by the absence of the marker gene function. Alternatively, a marker gene may be placed in tandem with the tie sequence under the control of the same or different promoter used to control the expression of the tie coding sequence. Expression of the marker in response to induction or selection indicates expression of the tie coding sequence.

In the third approach, transcriptional activity of the tie coding region may be assessed by hybridization assays. For example, polyadenylated RNA may be isolated and analyzed by Northern blotting using a probe homologous to the tie coding sequence or particular portions thereof. Alternatively, the total nucleic acid of the host cell may be extracted and assayed for hybridization to such probes.

In the fourth approach, the expression of tie may be assessed immunologically, for example, by Western blots, immunoassays such as radioimmunoprecipitation, enzyme-linked immunoassays and the like. The ultimate test of the success of the expression system, however, involves the detection of the biologically-active tie gene product. A cell-free media obtained from the cultured transfectant host cell may be assayed for tie activity when the gene product is secreted.

When the gene product is not secreted, cell lysates may be assayed for such activity. In either case, assays which measure ligand binding to tie or other bioactivities of tie may be used.

D. tie Derivatives, Analogs and Peptides

The production and use of derivatives, analogs, and peptides related to tie are also within the scope of the invention. Such derivatives, analogs, or peptides may have enhanced or diminished biological activities in comparison to native tie. tie-related derivatives, analogs, and peptides of the invention may be produced by a variety of means known in the art. Procedures and manipulations at the genetic and protein levels are within the scope of the invention. Peptide synthesis, which is standard in the art, may be used to obtain tie peptides. At the protein level, numerous chemical modifications may be used to produce tie like derivatives, analogs, or peptides by techniques known in the art, including but not limited to, specific chemical cleavage by endopeptidases (*e.g.* cyanogen bromides, trypsin, chymotrypsin, V8 protease, and the like) or exopeptidases, acetylation, formulation, oxidation, etc.

E. Anti-tie Antibodies

Also within the scope of the invention is the production of polyclonal and monoclonal antibodies which recognize tie or related proteins. Various procedures known in the art may be used for the production of polyclonal antibodies to epitopes of tie. For the production of antibodies, various host animals may be immunized by injection with tie, or a synthetic tie peptide, including but not limited to, rabbits, mice, and rats. Various adjuvants may be used to increase the immunological response, depending upon the host species, including but not limited, to Freund's (complete and incomplete) adjuvant, mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, oil emulsions, keyhole limpet

hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (*Bacillus* Calmette-Guerin) and *Corynebacterium parvum*.

A monoclonal antibody directed against an epitope of tie may be prepared by using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique originally described by Kohler and Milstein, *Nature*, *256*: 495-497 (1975), and the more recent human B-cell hybridoma technique of Kosbor *et al.*, *Immunology Today*, 4:72 (1983) and the EBV-hybridoma technique of Cole *et al.*, *Monoclonal Antibodies and Cancer Therapy*, 77-96 (Alan R. Liss, Inc. 1985).

Antibody fragments which contain the idiotype of the molecule may be generated by known techniques. For example, such fragments include, but are not limited to, the F(ab')2 fragment which may be produced by pepsin digestion of the antibody molecule; the Fab' fragments which may be generated by reducing the disulfide bridges of the F(ab')2 fragment; and the two Fab fragments which may be generated by treating the antibody molecule with papain and a reducing agent.

Antibodies to tie may find use in the qualitative and quantitative detection of mature tie and its precursor and subcomponent forms, in the affinity purification of tie polypeptides, and in the elucidation of tie biosynthesis, metabolism and function. Detection of tie tyrosine kinase activity may be used as an enzymatic means of generating and amplifying a tie-specific signal in such assays. Antibodies to tie may also be useful as diagnostic and therapeutic agents.

F. Uses of tie, tie-encoding Nucleic Acid Molecules and Anti-tie Antibodies

Compositions of the present invention may be applied to a wide variety of uses, including diagnostic and/or therapeutic uses of tie, tie analogs and derivatives, tie-encoding nucleic

acid molecules, antisense nucleic acid molecules and anti-tie antibodies.

Tie-encoding nucleic acid molecules or fragments thereof may be used as probes to detect and quantify mRNAs encoding tie. Assays which utilize nucleic acid probes to detect sequences comprising all or part of a known gene sequence are well-known in the art. Tie mRNA levels may indicate emerging and/or exiting neoplasias as well as the onset and/or progression of other human diseases. Therefore, assays which detect and quantify tie mRNA may be of considerable diagnostic value.

Anti-sense tie RNA molecules may be useful therapeutically to inhibit the translation of tie-encoding mRNAs where the therapeutic objective involves the elimination of the presence of tie or to downregulate its levels. Tie anti-sense RNA, for example, may be useful as a tie-antagonizing agent in the treatment of diseases for which tie is involved as a causative agent, for example due to its overexpression.

Additionally, tie anti-sense RNAs may be useful in elucidating tie functional mechanisms. Tie-encoding nucleic acid molecules may be used for the production of recombinant tie proteins and related molecules as separately discussed in this application.

Anti-tie antibodies may be used to diagnose and quantify tie in various contexts. For example, antibodies against various domains of tie may be used as a basis for tie immunoassays or immunohistochemical assessment of tie. Tyrosine kinase activity of tie may be useful in these assays as an enzymatic amplification reaction for the generation of a tie signal. Anti-tie antibodies may also be useful in studying the amount of tie on cell surfaces.

Antibodies may be produced which function as tie ligand agonists or antagonists whereby the regulation of tie activity becomes possible. Since tie apparently is located on endothelial surfaces facing the vascular lumen, introduction of tie extracellular domain, its fragments or analogs, ligands

or anti-tie extracellular domain antibodies into the bloodstream may allow the manipulation of tie activity and function *in vivo* with consequences on endothelial cell behavior and disease onset/progression.

The introduction and expression of genes in endothelial cells *in vivo* is envisioned and will allow further manipulation of tie activity via expression vectors producing tie or its various functional derivatives in endothelial cells. For example, when sufficiently overexpressed, receptor tyrosine kinases in which the tyrosine kinase domain has been specifically inactivated by *in vitro* mutagenesis function as dominant inhibitors of receptor function. Cloning of the tie promoter and regulatory sequences may allow targeting of gene expression mainly to endothelial cells *in vivo*.

G. Molecular Biology of Tie

Containing EGF-like, immunoglobulin-like, fibronectin-like and tyrosine kinase domains, tie belongs to four different gene superfamilies. A combination of motifs from all immunoglobulin, fibronectin and EGF-homology superfamilies in the extracellular domain is a unique feature among known receptor tyrosine kinases.

The EGF-like domain is a commonly found structural motif in cell surface and extracellular proteins involved in protein-protein interactions. Davis, *The New Biologist, 2*: 410-419, (1990). Many transmembrane receptors for either soluble or cell bound ligands contain EGF repeats. Furthermore, two of the six EGF repeats of thrombomodulin, an endothelial cell surface glycoprotein, have been reported to be responsible for thrombin binding, Stearns, *et al., J. Biol. Chem., 264*: 3352-3356, (1989), and the EGF domain of the lymph node homing receptor has been implicated in the adhesion of lymphocytes to high endothelial venules. Siegelman, *et al., Cell, 61*: 611-22, (1990). Also, some homeotic genes, such as Notch, delta, and crumbs of *Drosophila melanogaster* (Wharton, et al., *Cell, 43*: 567-581, 1985, Vissin, et al., *EMBO J., 6*: 3431-3440, 1987, Tepass, et al.,

Cell, 61: 787-799, 1990) and *lin12* and *glp-1* of *Caenorhabditis elegans* (Yochem, et al., *Nature, 335*: 547-550, 1988, Yochem and Greenwald, *Cell, 58*: 553-563 1989) encode large transmembrane proteins containing several EGF-like repeats. These proteins participate in several cell-fate decisions, requiring cell-cell communication. Genetic evidence further suggests, that the various EGF motifs function in different protein-protein interactions. Kelley, *et al., Cell, 51*: 539-548, (1987). Multiple EGF repeats are found also in extracellular matrix proteins mediating cell adhesion, such as laminin and tenascin. In addition, EGF repeats are a common motif in secreted proteins involved in blood clotting, including coagulation factors VII, IX, X, proteins C and S as well as tissue- and urokinase-type plasminogen activators. Furie and Furie, *Cell, 53*: 505-518, (1988). The EGF like domain of urokinase-type plasminogen activator has been reported to be responsible for its receptor binding. Appella, *et al., J. Biol. Chem., 262*: 4437-4440, (1987).

The EGF-like repeats of tie contain eight cysteine residues instead of the usual six. Although eight cysteines are also found in the EGF repeats of laminin, the tie repeats are clearly most related to each other. None of the repeats of tie contains the consensus sequence required for asparagine/aspartate s-hydroxylation and calcium binding. The finding of a tie cDNA clone which encoded a protein lacking the first of the EGF-like repeats further suggests that these domains are located in separate exons and that the repeat structure was presumably created by exon duplication in the course of the molecular evolution of the tie receptor tyrosine kinase. In addition, the observation of several tie mRNA forms in EAhy926 cells supports the notion that various forms of the tie receptor are produced, presumably due to differential splicing.

The immunoglobulin and fibfonectin superfamilies also comprise glycoproteins implicated in extracellular protein-protein interactions with either soluble or cell bound molecules. Williams and Barclay, *Ann. Rev. Immunol., 6*:

381-405, (1988). Many receptor tyrosine kinases such as PDGF, CSF-1 receptors, c-kit proto-oncogene as well as the FGF receptors contain Ig-like loops. Ullrich and Schlessinger, *Cell, 61*: 243-54, (1990). In many cases both immunoglobulin and fibronectin type III domains are found in the same protein. This type of multidomain structure has recently been reported to be present in some receptor tyrosine kinases. O'Bryan, *et al., Mol. Cell. Biol., 11*: 5016-5031, (1991); Rescigno, *et al., Oncogene, 6*: 1909-1913, (1991). As both immunoglobulin and FNIII repeats have been suggested to have a common evolutionary origin (Bazan, *Proc. Natl. Acad. Sci. USA, 87*: 6934-6938, 1990), it is interesting to note that repeat regions of the tie protein possess features of both of these classes. The presence of EGF, immunoglobulin, and fibronectin-like structural motifs in the extracellular domain of the tie protein suggests that the tie receptor might interact with several different extracellular molecules.

The regional localization of the tie gene at 1p33-p34 indicates that the tie locus is telomeric of the *jun* locus since the PB5-5 hybrid which is negative for tie is positive for *jun*. Haluska, *et al., Proc. Natl. Acad. Sci. USA, 85*: 2215-2218, (1988). The chromosomal region 1p32-p34 is involved in deletions in neuroblastoma, malignant lymphoma, glioma and other malignancies. Trent, *et al., Cytogenet. Cell Genet., 51*: 533-562, (1989).

Our earlier and present experiments indicate that the mRNA for the tie receptor is expressed only in few tumor cell lines in culture. In contrast, expression was evident in Northern blotting of all mouse and human fetal tissues studied. This pattern of expression is compatible with the possibility that the signal obtained from tissues is derived from endothelial cells, as suggested by the finding of tie mRNA in the EA.hy926 and PAEC endothelial cell lines as well as in primary cultured human endothelial cells. Furthermore, *in situ* hybridization analyses of tie expression in human as well as in mouse tissues indicate that tie mRNA is present in endothelial cells.

The above-stated findings on tie mRNA expression suggest that the tie expression product is characteristic of the bipotential hematopoietic cell lineage retaining erythroid and megakaryoblastic differentiation capacities as well as for the endothelial cell lineage. Several differentiation antigens shared between megakaryoblastic and endothelial cells have been shown to exist, one example being the platelet glycoprotein IIIa *Blood, 72*: 1478-1486, (1988); Kieffer, *et al., Blood, 72*: 1209-1215, (1988); Berridge, *et al., Blood, 66*: 76-85, (1985). The observed expression pattern of tie mRNA is rather intriguing as EGF motifs are a common theme of proteins controlling hemostasis as well as proteins mediating associations
with the endothelium.

EXAMPLE 1

Isolation and characterization of cDNA clones encoding tie

An oligo-dT primed human HEL cell cDNA library in bacteriophage lgt11 (A kind gift from Dr. Mortimer Poncz, Childrens Hospital of Philadelphia, PA; (Poncz, et al., *Blood, 69*: 219-223, 1987)) and a random primed human endothelial cell cDNA library (Clontech Cat. #1070b) were screened with the JTK14 cDNA fragment PCR-amplified from the reverse-transcribed polyadenylated RNA of K562 leukemia cells. Partanen, *et al., Proc. Natl. Acad. Sci. U S A, 87*: 8913-8917, (1990). Positive plaques were identified and purified as described in Sambrook, *et al.*, Molecular cloning - a laboratory manual, Cold Spring Harbor Laboratory Press, 1989. cDNA inserts of bacteriophage lambda were isolated as *EcoRI*-fragments and subcloned into GEM3Zf(+) plasmid (Promega). The entire tie protein coding region was isolated from both libraries. Two overlapping clones isolated from a HEL-library (HE11-1, nucleotides 62 to 3845 in Fig.1, and 12a, nucleotides 1 to 2446) were sequenced using the dideoxy chain termination method with oligonucleotide primers designed according to the sequences obtained. All portions of the cDNAs were sequenced on both strands. Sequence analyses were performed using the GCG

package programs (Devereux, et al., *Nucleic Acids Res., 12*: 387-395, 1984) and the Prosite program for Apple MacIntosh.

A 200 bp long tie cDNA fragment isolated by a PCR cloning method from K562 cell cDNA was used as a molecular probe to screen an oligo dT-primed human erythroleukemia cell cDNA library and a random-primed human endothelial cell cDNA library. Nucleotide sequence analysis of clones HE11-1 and 12a isolated from the HEL library revealed an open reading frame of 1138 amino acids (Fig. 1). The translational initiator, methionine, marked in Figure 1 is surrounded by a typical consensus sequence (Kozak, *Nucleic Acids Res., 12*: 857-872, 1984) and followed by a hydrophobic amino acid sequence characteristic of signal sequences for translocation into the endoplasmic reticulum. Beginning with amino acid residue 214 of the reading frame there is a region of 130 amino acid residues containing 24 cysteine residues. This region may be aligned into three repeated homologous domains containing eight cysteine residues each (Fig. 2A). Figure 2A also shows the comparison of the tie cysteine-rich domains with the epidermal growth factor (EGF) and CRIPTO growth factor proteins and the EGF-like repeats of laminin A chain, the Notch and Lin12 developmental control proteins of *Drosophila melanogaster* and *Caenorhabditis elegans*, respectively, and blood coagulation factor IXa. Significant structural similarities are present between tie and the EGF family, which allow the inclusion of the cysteine-rich repeats of tie in the EGF repeat family. However, the tie repeats are more closely related to each other than to other members of the EGF repeat family. This is particularly evident when examining the amino terminal ends of the repeats, whose three cysteine residues are not conserved in other EGF repeats (Fig. 2A). In addition to several tie cDNA molecules encoding three EGF repeats, a cDNA clone was isolated from HEL cell cDNA library, which lacked the first of the EGF repeats (marked between the arrowheads in Fig. 1) without otherwise affecting the reading frame. The amino-terminal region of the tie extracellular domain shows weak, but significant, homology to

the amino terminus of chicken N-CAM protein (Cunningham, et al., *Science, 236*: 799-806, 1987). As with N-CAM, a pair of cysteine residues surrounded by consensus motifs characteristic for the proteins of the immunoglobulin superfamily (Williams and Barclay, *Ann. Rev. Immunol., 6*: 381-405, 1988) is found in this region (Ig1 in Fig.1). In addition, two pairs of cysteine residues are located carboxyl terminal of the three EGF repeats. The amino acid sequence around the first cysteine pair shows additional homology to immunoglobulin domains (Ig2 in Fig.1). The extracellular region following the Ig2 domain (including one of the cysteine pairs) may be aligned into three repeats that are homologous to fibronectin type III (FNIII) repeats. The three repeats of the tie protein and their comparison with the FNIII repeats present in the human LAR phosphotyrosine phosphatase (Streuli, et al., *J. Exp. Med., 168*: 1553-1562, 1988) are shown in Fig. 2B. Interestingly, the second of these three repeats (FN2) contains a pair of cysteine residues as well as some other features of an immunoglobulin domain (Fig. 2B) and thus represents an intermediate of a FNIII repeat and an immunoglobulin domain.

Five consensus sites for potential N-linked glycosylation (NXS/T, X = any amino acid) may be distinguished in the extracellular domain. None of these appears in the EGF repeats. Amino acids 761-787 form a hydrophobic region of the sequence, which is likely to function as the transmembrane domain of the receptor, followed by several basic residues on the putative cytoplasmic side of the polypeptide. The juxtamembrane domain is 50 residues long before the beginning of a tyrosine kinase sequence homology at amino acid 837. With the interruption of homology in the kinase insert sequence of 14 aa (indicated by italics in the Fig. 1), this homology is first lost at the beginning of the 31 amino acid carboxyl terminal tail of the receptor. A search for related tyrosine kinase domains in the amino acid sequence database (Swissprot and NBRF) identified the FGFR-1, ret, c-fms, PDGFR and c-kit receptor tyrosine kinases as the closest homologs of

tie (about 40% amino acid sequence identity in the tyrosine kinase domain).

EXAMPLE 2

Preparation of Antisera

A tie cDNA fragment encoding 196 carboxyl terminal amino acids was inserted into pEX2 bacterial expression vector (Stanley and Luzio, *EMBO J.* 3: 1429-1434, 1984) using an internal *Xho*I site. The resulting s-galactosidase fusion protein was produced in bacteria and partially purified by preparative SDS-polyacrylamide gel electrophoresis. Polypeptide bands were cut out from the gel, minced, mixed with Freund's adjuvant, and used for immunization of rabbits. Antisera were used after the third booster immunization. A peptide corresponding to 15 amino acids from the carboxyl terminus of the predicted tie protein was synthesized and cross-linked by glutaraldehyde to keyhole limpet hemocyanin (KLH, Calbiochem). The immunizations were performed as in Example 1. Briefly, 7.5 mg carrier protein was dissolved in 0.5 ml of 0.1 M phosphate, pH 8.0, mixed with 7.5 mg of peptide and 5 ml of 20 mM glutaraldehyde was added. After mixing the solution, it was left for 15 min. at room temperature, after which 2.5 ml of glutaraldehyde was again added and the 15 min. incubation was repeated. Then, 0.1 ml of 1 M glycine, pH 6.0 was added to block unreacted glutaraldehyde and the stirring resumed for an additional 10 min. The product was dialyzed exhaustively against phosphate-buffered saline. For immunization, 1.25 mg of synthetic peptide-KLH conjugate in 0.5 ml PBS pH 7.5 was mixed with 0.5 ml complete Freund's adjuvant. The emulsion was delivered by subcutaneous injections, 0.1 ml in each of ten sites, into 3 month old New Zealand white rabbits. After biweekly intervals, the immunization was repeated with an identical quantity of immunogen. Serum was prepared from blood collected from auricular vein one week after the second and subsequent booster injections.

EXAMPLE 3

Expression of tie in COS cells

The full-length tie protein coding sequence (combined from two overlapping clones, HE11-1 and 12a) was inserted into the *Eco*RI site of an SV poly-mammalian expression vector (Stacey and Schnieke, *Nucleic Acids Res., 18*: 1829, 1990; construct SV14-2). The SV14-1 vector lacks the first seven amino acids from its signal sequence, but it is initiated from an ATG codon present in the SV-poly vector. The expression vectors (SV14-2, SV14-1) were introduced into COS-1 cells by the DEAE-dextran transfection method (McCutchan and Pagano, *J. Natl Cancer Inst., 41*: 351-357, 1968). Two days after transfection the cells were labelled for 4 hours with ^{35}S-methionine in the presence or absence of 10 g/ml tunicamycin. The cells were washed with PBS and scraped into immunoprecipitation buffer (10mM Tris pH 7.5, 50 mM NaCl, 0.5% sodium deoxycholate, 0.5% Nonidet P40, 0.1% SDS, 0.1 TIU/ml Aprotinin). The lysates were sonicated, centrifuged for 15' at 10 000 g, and incubated overnight on ice with 3 ml of the antisera. Protein A sepharose (Pharmacia) was added and the incubation was continued for 30' with rotation. The precipitates were washed four times with the immunoprecipitation buffer, once with PBS, and once with aqua before analysis in SDS-PAGE.

The structural predictions of the tie cDNA sequence were tested by cloning the full-length tie protein coding region into the *Eco*RI site of the pSVpoly expression vector (constructs pSV14-2 and pSV14-1), and these expression vectors were then transfected into COS cells. The proteins produced by these two constructs differ in their signal sequence as noted above, but the predicted mature protein products are identical. After two days, the cells were metabolically labelled and immunoprecipitated with antibodies generated against as s-galactosidase-tie fusion protein containing 195 carboxyl terminal amino acid residues of the predicted tie protein (antiserum HI), or against a 15 amino acid peptide corresponding to the tie carboxyl terminus (antiserum MI).

Fig. 3 shows analysis of the immunoprecipitated radioactive polypeptides by SDS-polyacrylamide gel electrophoresis. As can be seen from Fig. 3A, the HI immune serum precipitated some weakly labeled polypeptides from untransfected COS cells. These polypeptides were probably not related to tie because the COS cells do not express its mRNA.

Cells transfected with the pSV14 expression vector show an additional specific polypeptide of 117 kD (marked tie in figure 3). This tie polypeptide was not precipitated with the preimmune serum or the antiserum blocked with the immunogen. The 117 kD polypeptide was recognized also by the MI antiserum against a carboxyl terminal peptide (Fig. 3B). Immunoprecipitation of tie polypeptides from transfected COS cells metabolically labeled in the presence of tunicamycin to prevent N-linked glycosylation of proteins gave a specific polypeptide of approximately 105 kD apparent molecular weight (marked tie* in Fig. 3B).

EXAMPLE 4

Expression of tie in NIH3T3 cells

The full-length tie cDNA was subcloned under the control of Moloney murine leukemia virus long terminal repeat promoter. This expression vector was used to co-transfect NIH3T3 cells with the pSVneo1 marker plasmid and G418 resistant clones were analyzed for tie expression. Cells on one confluent plate were lysed in 2.5% SDS, 125mM Tris, pH 6.5 for immunoblot analysis. Cell lysates were electrophoresed on SDS-page and electroblotted on nitrocellulose membrane. The membrane was incubated with the anti-peptide antiserum against the tie carboxyterminus and bound antibodies were visualized using horseradish peroxidase conjugated swine anti-rabbit antiserum (Dako) and ECL reagents (Amersham). Tyrosine phosphorylated proteins were immunoprecipitated as described (Frackelton, et al., 1991, In T. Hunterand B. M. Sefton (ed.), Protein phosphorylation part B, Meth. Enzymol. 201:79-91). Briefly, cells on one confluent were lysed in extraction buffer (1% Triton X-100, 10mM Tris pH 7.6, 5mM

EDTA, 50mM NaCl, 100 M Sodium orthovanadate, 1mM PMSF) and the lysates were incubated with rotation for 2 hours on ice with agarose conjugated anti-phosphotyrosine antibodies (1G2-A, Oncogene Science). The immunoprecipitates were washed four times with extraction buffer, and tyrosine phosphorylated proteins were eluted with 1mM phenyl phosphate. Eluted proteins were analyzed with immunoblotting as described above.

The 117 kD tie protein was detected by immunoblotting with the antiserum raised against the peptide corresponding the tie carboxyterminus (Fig. 4). In addition, endogenous tie protein of a similar molecular weight was detected in PAE (porcine aortic endothelial) cells. The tie protein was also detected in anti-phosphotyrosine immunoprecipitates of the tie -transfected cells.

EXAMPLE 5

Chromosomal mapping of the tie locus

Metaphase spreads from normal human male peripheral blood buffy coat leukocytes were prepared and hybridized essentially as described in Harper and Saunders, *Chromosoma, 83*: 431-439, (1981). For *in situ* hybridization, about 1 mg of HE11-1 cDNA insert was labeled by nick translation using four ^{3}H-labeled NTPs to a specific activity of about 4-8x107cpm/mg. After hybridization, slides were washed in 50% formamide, 2 x SSC at 39°C, and exposed to Kodak NTB2 nuclear track emulsion for 12 days at 4°C. The slides were developed with Kodak Dektol developer and Kodafix solution, and chromosomes were first G-banded with Wright-Giemsa stain (Cannizarro and Emanuel, *Cytogenet. Cell Genet., 38*: 308-309), and if necessary, rebanded by the trypsin-Giemsa (GTG) technique.

In situ hybridization of radiolabeled tie probe to normal human metaphase chromosomes localized tie sequences to chromosome 1. A total of 317 chromosomally-localized grains were scored on 145 metaphases. Thirty-four percent (109/317) of the grains were on chromosome 1 with 69% (75/109) of chromosome 1 grains localized to 1p33-p34. Grain localization on chromosome 1 is illustrated schematically in Fig. 5, where

each dot represents 3 grains. This narrows the localization to 1p33 - p34, with the highest concentration of grains close to the border between bands 1p33 and p34. Chromosomal localization using a panel of somatic mouse-human hybrid cell lines also placed the tie locus to human chromosome 1.

EXAMPLE 6

Expression of the tie mRNA in leukemia cell lines and endothelial cells

The leukemia cell lines used in this study have been reported in several previous publications; K562 (Lozzio and Lozzio, *Blood, 45*: 321-334, 1975), HL-60 (Collins, et al., *Nature, 270*: 347-349, 1977), HEL (Martin and Papayannopuolou, *Science, 216*: 1233-1235, 1982), Dami (Greenberg, et al., *Blood, 72*: 1968-1977, 1988), MOLT-4 (Minowada, et al., *J. Natl. Cancer Inst., 49*: 891-895, 1972), Jurkat (Schwenk and Schneider, *Blut, 31*: 299-306, 1975), U937 Sundstrom and Nilsson, *Int. J. Cancer, 17*: 565-577, 1976), KG-1 (Koeffler and Golde, *Science, 200*: 1153-1154, 1978), JOK-1 (Andersson et al., 1982, In R. F. Revoltella (ed.), Expression of differentiated functions in cancer cells. p.239-245, Raven Press, New York), ML-2 (Gahmberg et al., 1985, In L. C. Andersson, *et al.* (ed.), Gene expression during normal and malignant differentiation. p. 107-123, Academic Press, London) and RC-2A (Bradley, *et al., Br. J. Haemat., 51*: 595, 1982). The leukemia cells were grown in RPMI containing 10% FCS and antibiotics. Dami cells were cultivated in Iscoves modified DMEM with 10% horse serum. A permanent hybrid cell line (EA.hy926) obtained by fusing first-passage human umbilical vein endothelial cells with the A549 lung carcinoma cells (Edgell, et al., *Proc. Natl. Acad. Sci. USA, 50*: 3734-3737) was cultured in DMEM-HAT medium containing 10% FCS and antibiotics. The PAE cells (a kind gift from Dr. Lena Claesson-Welsh, Ludwig Institute for Cancer Research, Uppsala, Sweden) were grown in Ham's F12 medium containing 10% FCS Poly(A)+ RNA was extracted from the cell lines as described in

Sambrook, et *al.*, Molecular cloning - a laboratory manual, Cold Spring Harbor Laboratory Press, 1989. Five grams of the Poly(A)+ RNA samples were electrophoresed in agarose gels containing formaldehyde and blotted using standard conditions (Sambrook, et al., *supra*). The insert of the HE11-1 cDNA clone was labelled by the random priming method and hybridized to the blots. Hybridization was carried out in 50% formamide, 5 x Denhardt's solution (100x Denhardt's solution comprises 2% each of Ficoll, polyvinylpyrrolidone and bovine serum albumin), 5 x SSPE (3M NaCl, 200mM NaH_2PO_4 . H_2O, 20 mM EDTA, pH 7.0), 0.1% SDS (sodium dodecyl sulphate), and 0.1 mg/ml of sonicated salmon sperm DNA at 42°C for 18-24 h. The filters were washed at 65°C in 1xSSC (150 mM NaCl, 15mM sodium citrate, pH 7.0), 0,1% SDS and exposed to Kodak XAR-5 film.

Figure 6 shows the results of analysis of tie mRNA expression in ten leukemia cell lines. Only the HEL erythroleukemia cells, KG-1 myeloid leukemia cells and Dami megakaryoblastic leukemia cells expressed a 4.4 kb tie mRNA, as detected with the 3.8 kb tie cDNA probe. The Jurkat and MOLT-4 T-cell leukemias, as well as HL-60 promyelocytic leukemia, U937 and RC-2A monocytic leukemias, JOK-1 hairy cell leukemia and ML-2 myeloid leukemia cells were negative for the tie mRNA. The tie mRNA was also induced after TPA treatment of the K562 cells, when the cells undergo megakaryoblastoid differentiation. Interestingly, porcine aortic endothelial cells (PAE) as well as a hybrid human endothelial cell line, EA.hy926, which has been reported to express several endothelial markers in vitro (Edgell, et al., *Proc. Natl. Acad. Sci. USA, 50*: 3734-3737, 1983, Emeis and Edgell, *Blood, 71*: 1669-1675, 1988), expressed tie mRNA abundantly (Fig. 7). The EA.hy926 cell line was created by the fusion of human umbilical vein endothelial cells with A549 lung carcinoma cell line. The A549 cells were negative for tie mRNA expression. In addition to the 4.4 kb mRNA, the EA.hy926 cells expressed tie mRNA species of 3.9, 4.2 and 4.7 kb. The results of Northern blot analyses of the tie mRNA expression in cell lines are summarized in table 1.

EXAMPLE 7

Expression of tie in blood vessels

Selected fragments of cloned human tie cDNA outside of the tyrosine kinase domain exhibiting a low degree of homology to other receptor tyrosine kinases were used as in situ hybridization probes to detect the tie mRNA. Specifically, we used a *Sma*I fragment (nucleotides 268-1767) of the full-length cDNA clone corresponding to the extracellular domain of the tie clone further digested to smaller fragments with *Pst*I and *Sal*I. The probe was labeled with ^{35}S-deoxy(thio)ATP for the *in situ* hybridizations (Feinberg and Vogelstein, *Anal. Biochem., 132*: 6-13, 1983). Fragments of 100-790-bp generated by *Bgl*I of bacteriophage Lambda DNA were labeled similarly and used as a negative control probe. All specimens from fetal abortuses were obtained with permission of the joint ethical committee of the University Central Hospital and University of Turku (Turku, Finland). *In situ* hybridizations were carried out as described previously (Sandberg and Vuorio, *J. Cell. Biol.*, 104: 1077-1084). In brief, tissue samples of 15-19-week human fetuses obtained from therapeutic abortions were fixed with formaline and embedded in paraffin for sectioning. The sections were pretreated with proteinase K and HCl and acetylated. The hybridizations were carried out at 42°C for 24 h using ^{35}S-deoxy(thio)ATP-labeled probes, followed by washing, autoradiography at +4°C for 5-25 days, and staining of the sections with hematoxylin.

Tie mRNA expression in tissues was studied by mRNA *in situ* hybridization of 15-19 week old human fetal tissues. In agreement with the tie expression in endothelial cell lines, tie mRNA was seen to be located in the walls of medium and large vessels of the kidney (Fig. 8). Labelled Lambda DNA used as a negative control provided no detectable hybridization signal over the background.

EXAMPLE 8

Analysis of tie mRNA in mouse embryos

Approximately 106 plaques from two lgt10 libraries (a kind gift of Dr. Brigitte Galliot, Zentrum fur Molekularbiologie Heidelberg, Germany) prepared from 10- and 11-day post coitum (p.c.) mouse embryonic mRNA were screened with a *Sma*I fragment (nucleotides 155 - 1765 ; this cDNA encodes the first immunoglobulin domain and EGF-like domains I - III of the extracellular part of the tie receptor) and 3.8 kb *Eco*R1 fragments of human tie receptor cDNA (Partanen et al., *Mol. Cell Biol.*, in press). These cDNA segments have only little homology with other known genes. The probe was labelled with [a-^{35}P]dCTP by the random priming method. The nitrocellulose replicas of each phage-infected plate were hybridized in 50 % deionized formamide, 5 x Denhardt's solution, 5xSSPE, 0.1 % SDS and 100 mg/ml ssDNA. Seven positive clones were purified out of which four were subcloned into pGEM 3Zf(+) (Promega) and sequenced. DNA sequencing was performed by the dideoxy chain termination method of Sanger,.et *al.*, *Proc. Natl. Acad. Sci. USA* 74: 5463-5467, (1977), using a modified T7 DNA polymerase (Sequenase TM, U.S. Biochemical). Sequences were generated from both ends of subcloned restriction fragments using universal pGEM sequencing primers. The internal sequence of large fragments as well as the complementary strands of all fragments were determined using oligonucleotide primers synthesized according to sequence information on preceding sequences. Two nonoverlapping inserts of the mouse tie cDNA plasmid clones, designated D10E5 and 1C1D (Fig. 10) were used as hybridization probes. Mouse embryos of 8 - 14 -days p.c. were derived from matings of CBA and NMR mice. Embryonic age was calculated from the day on which the copulation plug was detected as day 0 (estimated copulation time 2 a.m.). Pregnant mice were killed by cervical dislocation, the embryos were removed and transferred immediately via phosphate buffered saline (PBS) into 4 % paraformaldehyde in PBS, pH 7.2. The embryos were fixed for 18 h at 4°C, dehydrated,

embedded in wax (Fisher Scientific Co) and cut into 5-6 mm sections. Isolated mouse organs were treated similarly. Total RNA was isolated from adult mouse organs and developing embryos according to Chirgwin, *et al.*, *Biochemistry, 18*: 5294-5299. Poly(A)+ RNA (5 g) and total RNA (20 g) were electrophoresed in 0.8 % agarose gels containing formaldehyde and blotted into Hybond-N (Amersham) filters using standard conditions. After transfer, the filters were exposed to ultraviolet radiation for 4 minutes, hybridized and washed in stringent conditions (Sambrook, *et al.*, Molecular cloning - a laboratory manual, Cold Spring Harbor Laboratory Press, 1989). *In situ* hybridization of sections was performed according to Wilkinson, *et al.*, *Development 99*: 493-500 (1987) with the following modifications: 1) instead of toluene, xylene was used before embedding in paraffin wax, 2) 5 - 6 mm sections were cut, placed on a layer of diethyl pyrocarbonate-treated (DEPC) water on the surface of glass slides pretreated with 2% 3-triethoxysilylpropylamine (TESPA) (Sigma), 3) alkaline hydrolysis of the probes was omitted, 4) the hybridization mixture contained 60 % deionized formamide, 5) the high stringency wash was for 80 minutes at 65°C in a solution containing 50 mM DTT and 1 x SSC, 6) the sections were covered with NTB-2 emulsion (Kodak) and stored at 4°C. After an exposure time of about 14 days the slides were developed for 2.5 min. in a Kodak D-19 developer and fixed for 5 min. with Unifix (Kodak). The sections were stained with 0.02 % toluidine blue in water. Control hybridizations with sense strand and RNAse A-treated sections did not give a specific signal above background. Immunoperoxidase staining was done using human monoclonal anti-factor VIII antibodies and standard techniques.

Total RNA and polyadenylated RNA were isolated from various human fetal and adult tissues as well as mouse tissues and subjected to Northern blotting and hybridization with the tie cDNA probes. Figure 11A shows that all fetal human tissues tested contain a 4.4 kb tie mRNA. In polyadenylated RNA from human adult tissues the tie signal is most prominent

in the highly vascularized lung, placenta and heart, but a weaker signal can also be recognized in other tissues as well, particularly in long exposure of the autoradiogram (Fig. 11B) The expression of tie begins very early; from 9 to 10 days of gestation tie is expressed weakly, then the number of tie transcripts increases (maximum at 14 days gestation). The newborn and postnatal mice have lower amounts of tie mRNA. Sagittal sections of 12 day p.c. mouse embryos were hybridized with the antisense and sense RNA transcribed from the insert of the 1C1D plasmid. Figure 12A shows the brightfield image of a representative section probed with antisense RNA. Figure 12A illustrates that the autoradiographic grains decorate the linings of major blood vessels. These signals were, however, better visualized in the darkfield microscopy of the same section (Fig. 12B). This result demonstrates that the tie mRNA is ubiquitously expressed in all vessels. The cells responsible for tie expression were endothelial cells as shown by Factor VIII immunostaining, which is specific to endothelial cells. The sense probe did not give signals above background, as can be seen from Fig. 12C. The tie hybridization signal in a 8-day p.c. mouse placenta (Fig. 13A) was very similar and practically superimposable with the pattern of factor VIII staining of adjacent sections (Fig. 13B).

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Partanen, Juha
Armstrong, Elina
Mäkelä, Tomi
Korhonen, Jaana
Alitalo, Kari

(ii) TITLE OF INVENTION: Tie, A Novel Endothelial Cell Recptor Tyrosine Kinase

(iii) NUMBER OF SEQUENCES: 4

(iv) CORRESPONDENCE ADDRESS:

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(C) CITY: 00510 Helsinki

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(F) ZIP: 00510

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

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(E) COUNTRY: Finland

(F) ZIP: 00120

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1138 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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Gln Ala Cys Arg Glu Gly Arg Phe Gly Gln Ser Cys Gln Glu Gln Cys
            260                 265                 270

Pro Gly Ile Ser Gly Cys Arg Gly Leu Thr Phe Cys Leu Pro Asp Pro
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Glu Ala

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1094 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Gly Ala Gly Arg Gly Ser Asp Ala Trp Gly Pro Pro Leu Leu Leu Glu
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Arg Leu Ile Val Arg Ala Cys Arg Glu Gly Arg Phe Gly Gln Ser Cys
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Gln Glu Gln Cys Pro Gly Ile Ser Gly Cys Arg Gly Leu Thr Phe Cys
225                 230                 235                 240

Leu Pro Asp Pro Tyr Gly Cys Ser Cys Gly Ser Gly Trp Arg Gly Ser
                245                 250                 255

Gln Cys Gln Glu Ala Cys Ala Pro Gly His Phe Gly Ala Asp Cys Arg
            260                 265                 270

Leu Gln Cys Gln Cys Gln Asn Gly Gly Thr Cys Asp Arg Phe Ser Gly
        275                 280                 285

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    290                 295                 300

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Glu Thr Met Pro Arg Ile Asn Cys Ala Ala Ala Gly Asn Pro Phe Pro
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        355                 360                 365

Val Pro Arg Leu Val Leu Ala Asp Ser Gly Phe Trp Glu Cys Arg Val
    370                 375                 380

Ser Thr Ser Gly Gly Gln Asp Ser Arg Arg Phe Lys Val Asn Val Lys
385                 390                 395                 400

Val Pro Pro Val Pro Leu Ala Ala Pro Arg Leu Leu Thr Lys Gln Ser
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580 585 590

Val Leu Leu Pro Pro Ser Gly Pro Pro Ala Pro Arg His Leu His Ala
595 600 605

Gln Ala Leu Ser Asp Ser Glu Ile Gln Leu Thr Trp Lys His Pro Glu
610 615 620

Ala Leu Pro Gly Pro Ile Ser Lys Tyr Val Val Glu Val Gln Val Ala
625 630 635 640

Gly Gly Ala Gly Asp Pro Leu Trp Ile Asp Val Asp Arg Pro Glu Glu

645 650 655

Thr Ser Thr Ile Ile Arg Gly Leu Asn Ala Ser Thr Arg Tyr Leu Phe
660 665 670

Arg Met Arg Ala Ser Ile Gln Gly Leu Gly Asp Trp Ser Asn Thr Val
675 680 685

Glu Glu Ser Thr Leu Gly Asn Gly Leu Gln Ala Glu Gly Pro Val Gln
690 695 700

Glu Ser Arg Ala Ala Glu Glu Gly Leu Asp Gln Gln Leu Ile Leu Ala
705 710 715 720

Val Val Gly Ser Val Ser Ala Thr Cys Leu Thr Ile Leu Ala Ala Leu
725 730 735

Leu Thr Leu Val Cys Ile Arg Arg Ser Cys Leu His Arg Arg Arg Thr
740 745 750

Phe Thr Tyr Gln Ser Gly Ser Gly Glu Glu Thr Ile Leu Gln Phe Ser
755 760 765

Ser Gly Thr Leu Thr Leu Thr Arg Arg Pro Lys Leu Gln Pro Glu Pro
770 775 780

Leu Ser Tyr Pro Val Leu Glu Trp Glu Asp Ile Thr Phe Glu Asp Leu
785 790 795 800

Ile Gly Glu Gly Asn Phe Gly Gln Val Ile Arg Ala Met Ile Lys Lys
805 810 815

Asp Gly Leu Lys Met Asn Ala Ala Ile Lys Met Leu Lys Glu Tyr Ala
820 825 830

Ser Glu Asn Asp His Arg Asp Phe Ala Gly Glu Leu Glu Val Leu Cys
835 840 845

Lys Leu Gly His His Pro Asn Ile Ile Asn Leu Leu Gly Ala Cys Lys
850 855 860

Asn Arg Gly Tyr Leu Tyr Ile Ala Ile Glu Tyr Ala Pro Tyr Gly Asn
865 870 875 880

Leu Leu Asp Phe Leu Arg Lys Ser Arg Val Leu Glu Thr Asp Pro Ala

885 890 895

Phe Ala Arg Glu His Gly Thr Ala Ser Thr Leu Ser Ser Arg Gln Leu
900 905 910

Leu Arg Phe Ala Ser Asp Ala Ala Asn Gly Met Gln Tyr Leu Ser Glu
915 920 925

Lys Gln Phe Ile His Arg Asp Leu Ala Ala Arg Asn Val Leu Val Gly
930 935 940

Glu Asn Leu Ala Ser Lys Ile Ala Asp Phe Gly Leu Ser Arg Gly Glu
945 950 955 960

Glu Val Tyr Val Lys Lys Thr Met Gly Arg Leu Pro Val Arg Trp Met
965 970 975

Ala Ile Glu Ser Leu Asn Tyr Ser Val Tyr Thr Thr Lys Ser Asp Val
980 985 990

Trp Ser Phe Gly Val Leu Leu Trp Glu Ile Val Ser Leu Gly Gly Thr
995 1000 1005

Pro Tyr Cys Gly Met Thr Cys Ala Glu Leu Tyr Glu Lys Leu Pro Gln
1010 1015 1020

Ala Asp Arg Met Glu Gln Pro Arg Asn Cys Asp Asp Glu Val Tyr Glu
1025 1030 1035 1040

Leu Met Arg Gln Cys Trp Arg Asp Arg Pro Tyr Glu Arg Pro Pro Phe
1045 1050 1055

Ala Gln Ile Ala Leu Gln Leu Gly Arg Met Leu Glu Ala Arg Lys Ala
1060 1065 1070

Tyr Val Asn Met Ser Leu Phe Glu Asn Phe Thr Tyr Ala Gly Ile Asp
1075 1080 1085

Ala Thr Ala Glu Glu Ala
1090

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3845 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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GCCAACCTGC GGCTCACGGA CCCCCAGCGC TTCTTCCTGA CTTGCGTGTC TGGGGAGGCC 180

GGGGCGGGGA GGGGCTCGGA CGCCTGGGGC CCGCCCCTGC TGCTGGAGAA GGACGACCGT 240

ATCGTGCGCA CCCCGCCCGG GCCACCCCTG CGCCTGGCGC GCAACGGTTC GCACCAGGTC 300

ACGCTTCGCG GCTTCTCCAA GCCCTCGGAC CTCGTGGGCG TCTTCTCCTG CGTGGGCGGT 360

GCTGGGGCGC GGCGCACGCG CGTCATCTAC GTGCACAACA GCCCTGGAGC CCACCTGCTT 420

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GACTGGCATG AAGCCCAGGA TGGGCGGTTC CTGCTGCAGC TCCCAAATGT GCAGCCACCA 600

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CGGCTCATCG TGCGGGGTTG TGGGGCTGGG CGCTGGGGGC CAGGCTGTAC CAAGGAGTGC 720

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CACCTCCACG CCCAGGCCCT CTCAGACTCC GAGATCCAGC TGACATGGAA GCACCCGGAG 2040

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GACCCACTGT GGATAGACGT GGACAGGCCT GAGGAGACAA GCACCATCAT CCGTGGCCTC 2160

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GCTGACCGCA TGGAGCAGCC TCGAAACTGT GACGATGAAG TGTACGAGCT GATGCGTCAG 3300

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(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3713 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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GCCAACCTGC GGCTCACGGA CCCCCAGCGC TTCTTCCTGA CTTGCGTGTC TGGGGAGGCC 180

GGGGCGGGGA GGGGCTCGGA CGCCTGGGGC CCGCCCCTGC TGCTGGAGAA GGACGACCGT 240

ATCGTGCGCA CCCCGCCCGG GCCACCCCTG CGCCTGGCGC GCAACGGTTC GCACCAGGTC 300

ACGCTTCGCG GCTTCTCCAA GCCCTCGGAC CTCGTGGGCG TCTTCTCCTG CGTGGGCGGT 360

GCTGGGGCGC GGCGCACGCG CGTCATCTAC GTGCACAACA GCCCTGGAGC CCACCTGCTT 420

CCAGACAAGG TCACACACAC TGTGAACAAA GGTGACACCG CTGTACTTTC TGCACGTGTG 480

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CGGCTCATCG TGCGGGCCTG CAGAGAGGGC CGTTTTGGGC AGAGCTGCCA GGAGCAGTGC 720

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GCAGCTGCAG GGAACCCCTT CCCCGTGCGG GGCAGCATAG AGCTACGCAA GCCAGACGGC 1080

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GTGCCCCGCT TGGTTCTTGC GGACAGTGGG TTCTGGGAGT GCCGTGTGTC CACATCTGGC 1200

GGCCAAGACA GCCGGCGCTT CAAGGTCAAT GTGAAAGTGC CCCCCGTGCC CCTGGCTGCA 1260

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CCCCCTGCAC ACGTGCTTCT GCCCCCCAGT GGGCCTCCAG CCCCCCGACA CCTCCACGCC 1860

CAGGCCCTCT CAGACTCCGA GATCCAGCTG ACATGGAAGC ACCCGGAGGC TCTGCCTGGG 1920

CCAATATCCA AGTACGTTGT GGAGGTGCAG GTGGCTGGGG GTGCAGGAGA CCCACTGTGG 1980

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TCCAAGATTG CAGACTTCGG CCTTTCTCGG GGAGAGGAGG TTTATGTGAA GAAGACGATG 2940

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GTTCAGCTGC CCCACAGGTG TGTTTCCCAT CCCACTGCTC CCCCAACACA AACCCCCACT 3600

CCAGCTCCTT CGCTTAAGCC AGCACTCACA CCACTAACAT GCCCTGTTCA GCTACTCCCA 3660

CTCCCGGCCT GTCATTCAGA AAAAAATAAA TGTTCTAATA AGCTCCAAAA AAA 3713

What is claimed is:

1. An isolated nucleotide sequence comprising a sequence encoding the protein sequence of tie receptor tyrosine kinase or a functional derivative or a fragment thereof.

2. An isolated nucleotide sequence according to claim 1 encoding a tie precursor comprising the nucleotide sequence substantially as depicted in SEQ ID NO: 1 from about nucleotide number 1 to about nucleotide number 3845.

3. An isolated nucleotide sequence according to claim 1 encoding a tie precursor comprising the nucleotide coding sequence substantially as depicted in SEQ ID NO: 1 from about nucleotide number 37 to about nucleotide number 3845.

4. An isolated nucleotide sequence according to claim 1 encoding a mature tie comprising the nucleotide coding sequence substantially as depicted in SEQ ID NO: 1 from about nucleotide number 100 to about nuoleotide number 3845.

5. A recombinant-DNA-molecule which comprises a nucleotide sequence selected from the group consisting of the nucleotide sequences according to any of the claims 1 to 4.

6. The recombinant-DNA-molecule according to claim 5, wherein said nucleotide sequence is operably linked to a suitable expression control sequence.

7. The recombinant-DNA-molecule according to claim 6, wherein said expression control sequence renders the said recombinant-DNA-molecule capable of expressing the said nucleotide sequence.

8. The recombinant-DNA-molecule according to claim 6, wherein said expression control sequence renders the said recombinant DNA molecule capable of expressing antisense RNA to the said nucleotide sequence.

9. A host cell transformed with the recombinant-DNA-molecule of claim 5.

10. The host cell according to claim 9, wherein said cell is a eukaryotic cell.

11. The host cell according to claim 10, wherein said cell is a mammalian cell.

12. A substantially pure tie protein or a functional derivative or a fragment thereof.

13. The substantially pure tie protein according to claim 12, wherein the tie protein is a tie precursor comprising the amino acid sequence substantially as depicted in SEQ ID NO: 1 from about amino acid residue number 1 to about amino acid residue number 1138.

14. The substantially pure tie protein according to claim 12, wherein the tie protein is a mature tie comprising the amino acid sequence substantially as depicted in SEQ ID NO: 1 from about amino acid residue number 22 to about amino acid residue number 1138.

15. The substantially pure tie protein according to claim 12, wherein said protein is human tie.

16. The substantially pure tie protein according to claim 12, wherein said protein is a recombinantly-produced tie.

17. The substantially pure tie protein according to claim 16, wherein said tie is produced in a mammalian cell culture.

18. A process for producing recombinant tie protein, which process comprises:

1) isolating nucleotide sequence encoding the said tie protein,

2) constructing an expression vector by introducing the nucleotide sequence into an appropriate cloning vector,

3) transforming appropriate host cells with said expression vector,

4) culturing said host cells, and

5) isolating the tie product.

19. The process for producing the recombinant tie according to claim 18, wherein said tie is human tie.

20. The process for producing the recombinant tie according to claim 18, wherein said host cells are mammalian cells.

21. The process according to claim 18, wherein the nucleotide seguence encoding tie comprises the nucleotide sequence substantially as depicted in SEQ ID NO: 1 from about nucleotide number 1 to about nucleotide number 3845.

22. The process according to claim 18, wherein the nucleotide seguence encoding tie comprises the nucleotide coding sequence substantially as depicted in SEQ ID NO: 1 from about nucleotide number 37 to about nucleotide number 3845.

23. The process according to claim 18, wherein the nucleotide seguence encoding tie comprises the nucleotide coding sequence substantially as depicted in SEQ ID NO: 1 from

about nucleotide number 100 to about nucleotide number 3845.

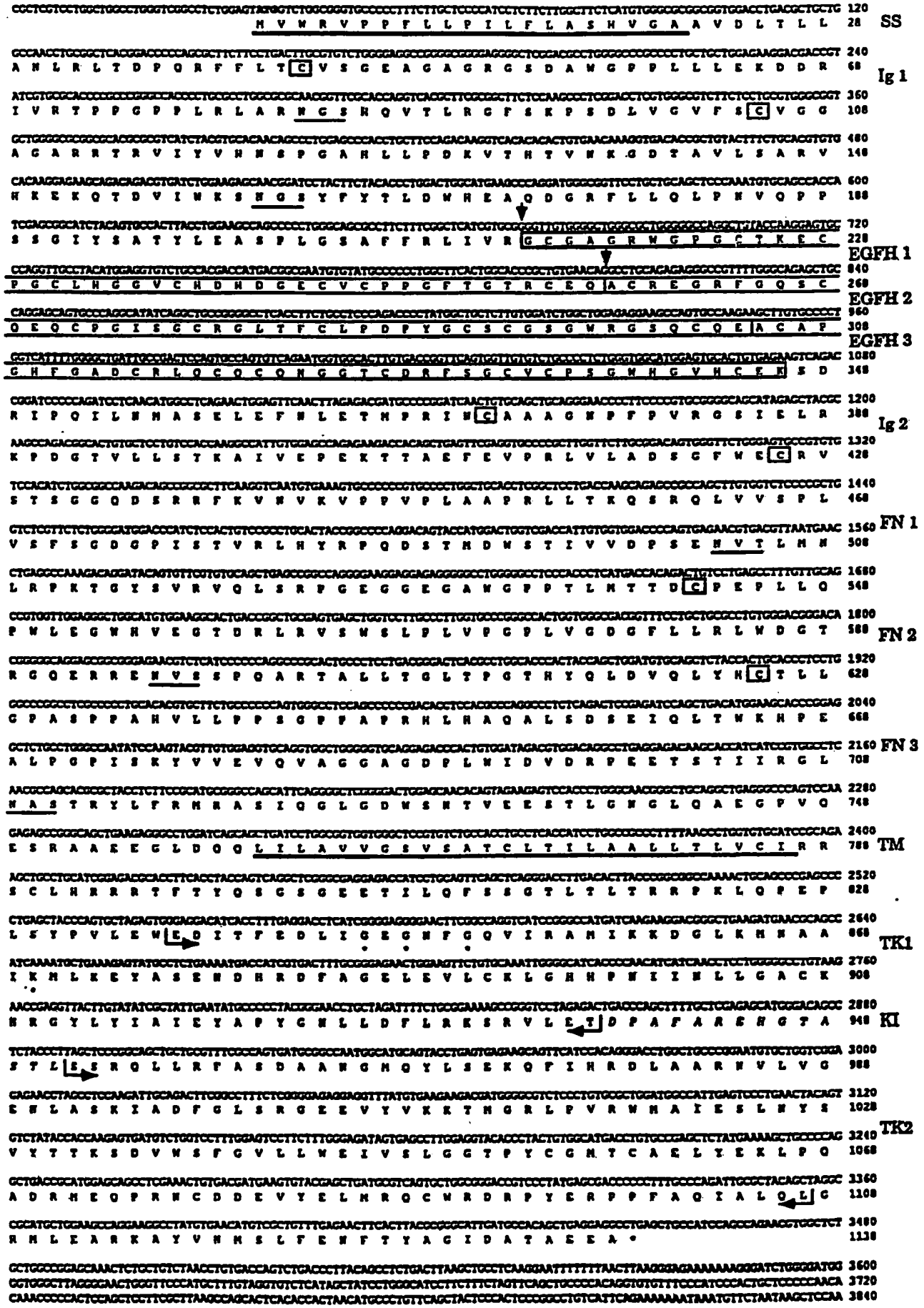
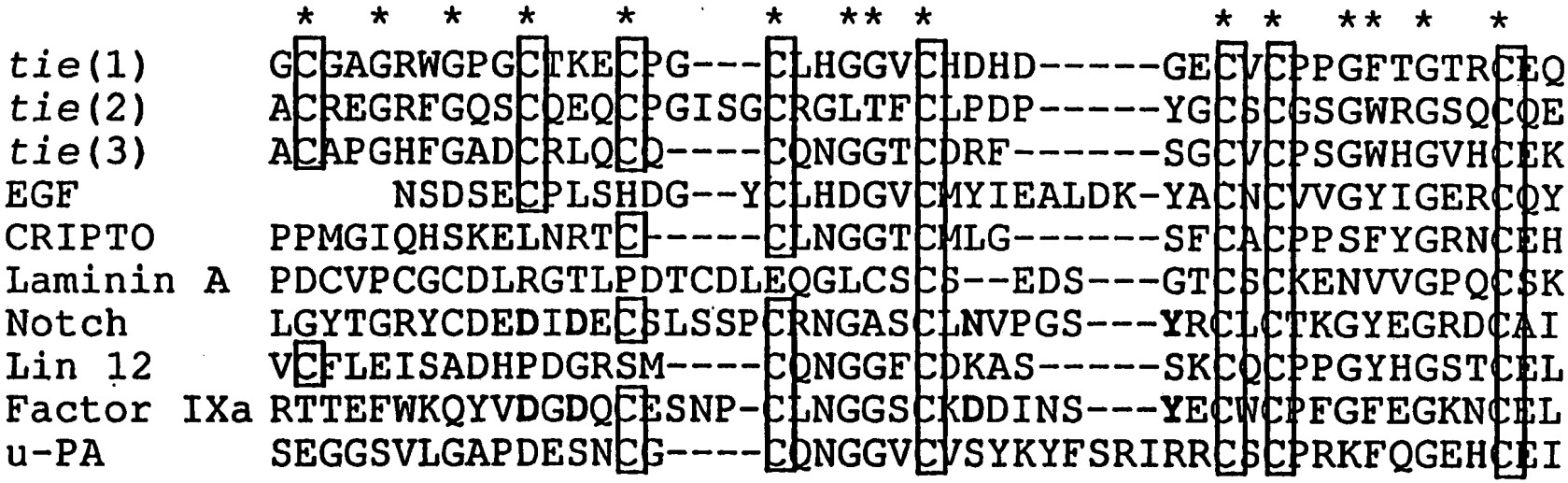


FIG 1

FIG. 2A

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          :|:| ::| :: |:::::: : |:  : ::::|:: ::||:::::: : ::  :::: :: :|:| ::|: || |:  : | : : ::
LAR(iv)   QVTVKALPKPPIDLVVTETTATSVTLTWDSGNSEPVTYYGIQYRAAGTEGPFQEVDGVATTRYSIGGLSPFSEYAFRV-LAVNSIGRGPPSEAVRA     396

                  v   c          w                                                          d g y c
tie(FN2)  MTTDCPEPLLQPWLEGWHVEGTDRLRVSWSLPLVPGPLVGDGFLLRLWDGTRGQERRENVSSPQARTALLTGLTPGTHYQLDVQLYHCTLLGPASPPAHV 637
          :|:: : : :::::::: ::::::| | |: |  |: || : :    |: | :: ::: :: ::  : :::| ||: | | |  :  :  ||:||: :|
LAR(v)    RTGEQAPSSPPRRVQARMLSASTML-VQWEPPEEPNGLVRGYRVYYTPDSRRPPNAWHKHNTDAGLLTTVGSLLPGITYSLRVLAFTAVGDGPPSPTIQV 495

tie(FN3)  LLPPSGPPAPRHLHAQALSDSEIQLTWKHPEALPGPISKYVVEVQVAGGAGDPLWIDVDRPEETSTIIRGLNASTRYLFRMRASIQ-GLGDWSNTVEEST 736
            ::: |::| :::|:: ||: |||:|  |   :::| :| :  :|:::::: :: |:  ::| :: :|:::| | |:: |: : |:| :: |:|::|
LAR(vi)   KTQQGVPAQPADFQAEVESDTRIQLSWLLPP--QERIIMYELVYWAAEDEDQQHKVTFDP--TSSYTLEDLKPDTLYRFQLAARSDMGVGVFTPTIEART 591
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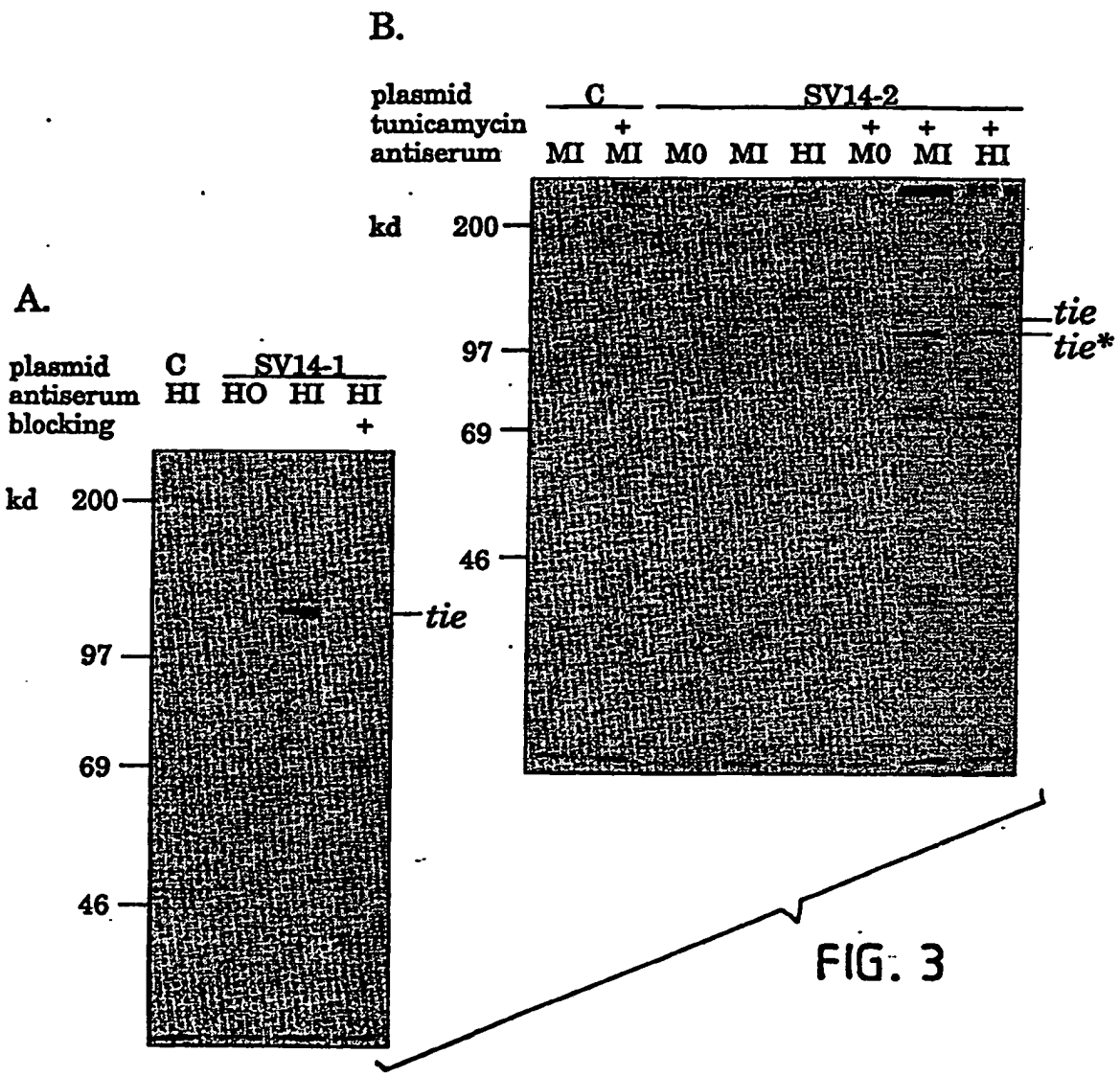
FIG. 2B


B.
plasmid C SV14-2
tunicamycin + + + +
antiserum MI MI MO MI HI MO MI HI
kd 200
97
69
46
tie
tie*
A.
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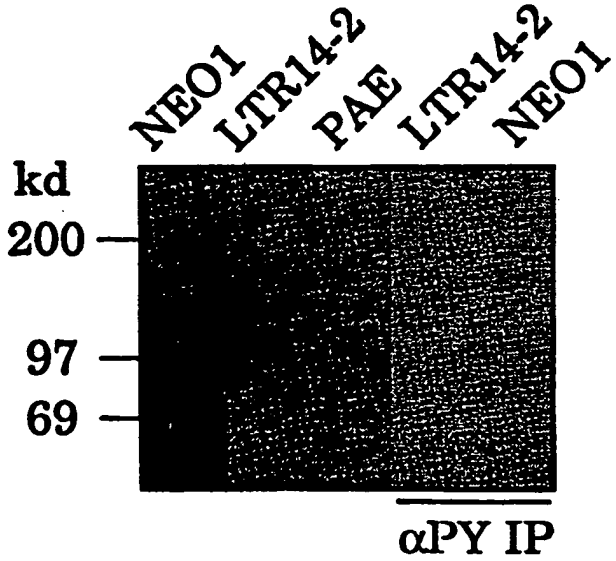
blocking +
kd 200
97
69
46
tie

FIG. 3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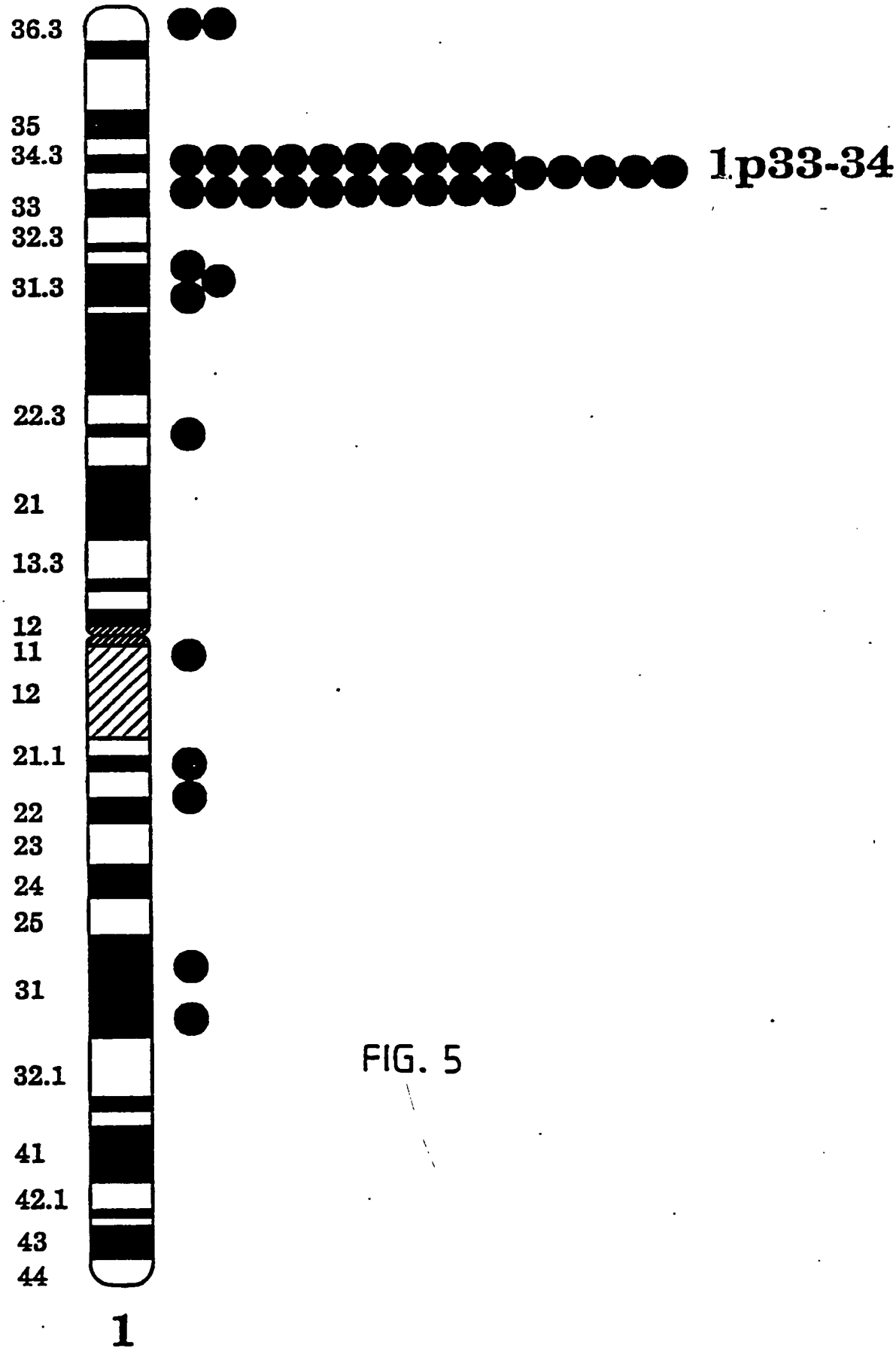
LTR14-2
PAE
LTR14-2
NEO1
kd
200
97
69
αPY IP

FIG. 4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44
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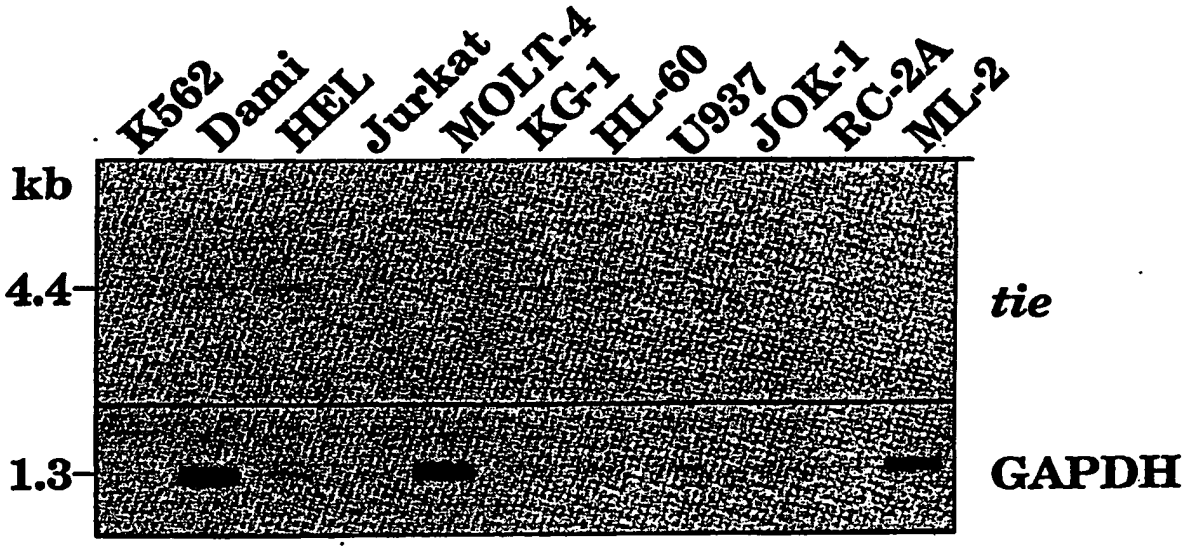
1p33-34

FIG. 5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Dami
HEL
Jurkat
MOLT-4
KG-1
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U937
JOK-1
RC-2A
ML-2
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4.4
1.3
tie
GAPDH

FIG. 6

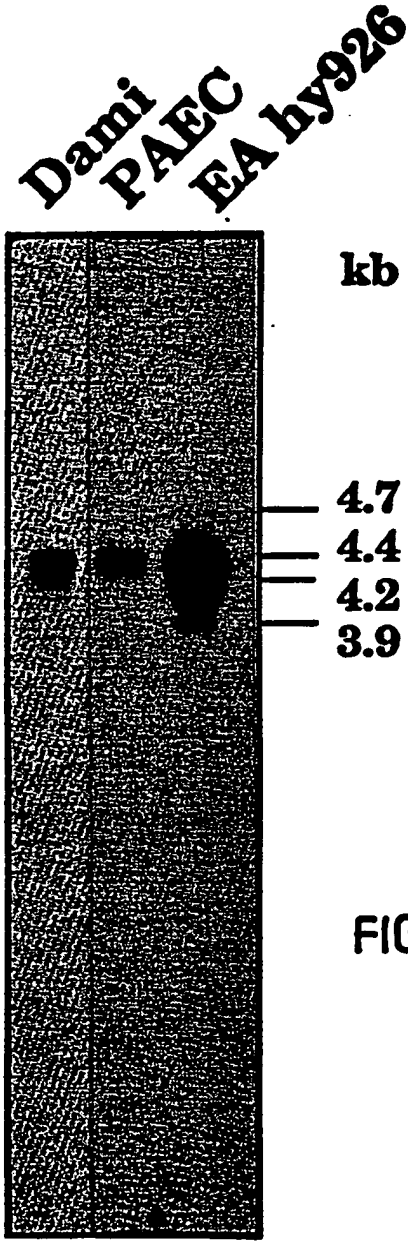
Dami
PAEC
EA hy926
kb
4.7
4.4
4.2
3.9

FIG. 7

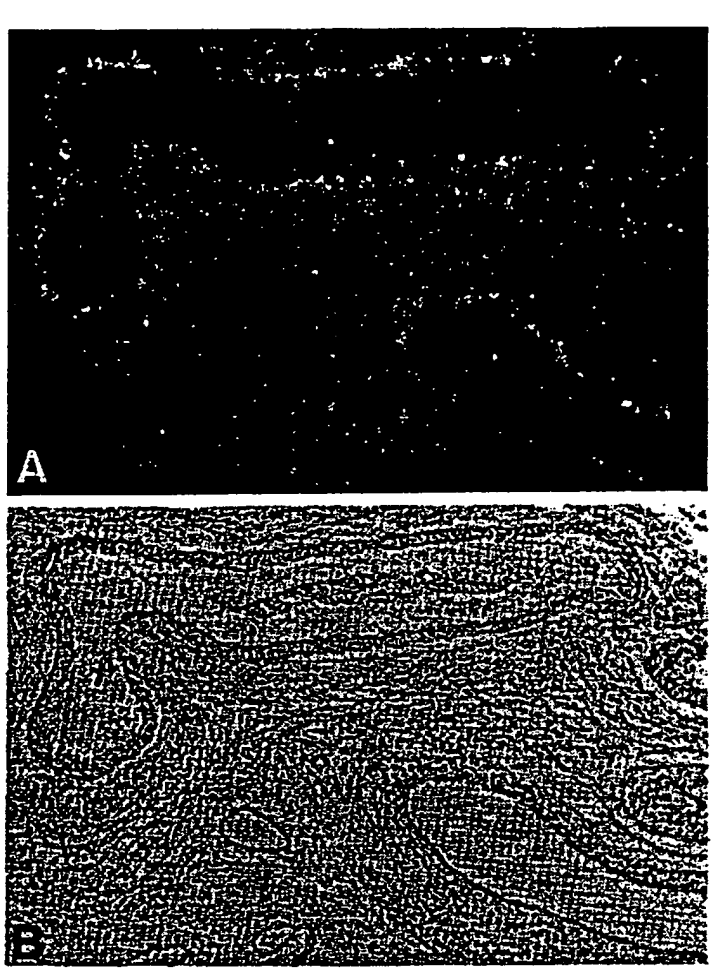

FIG. 8

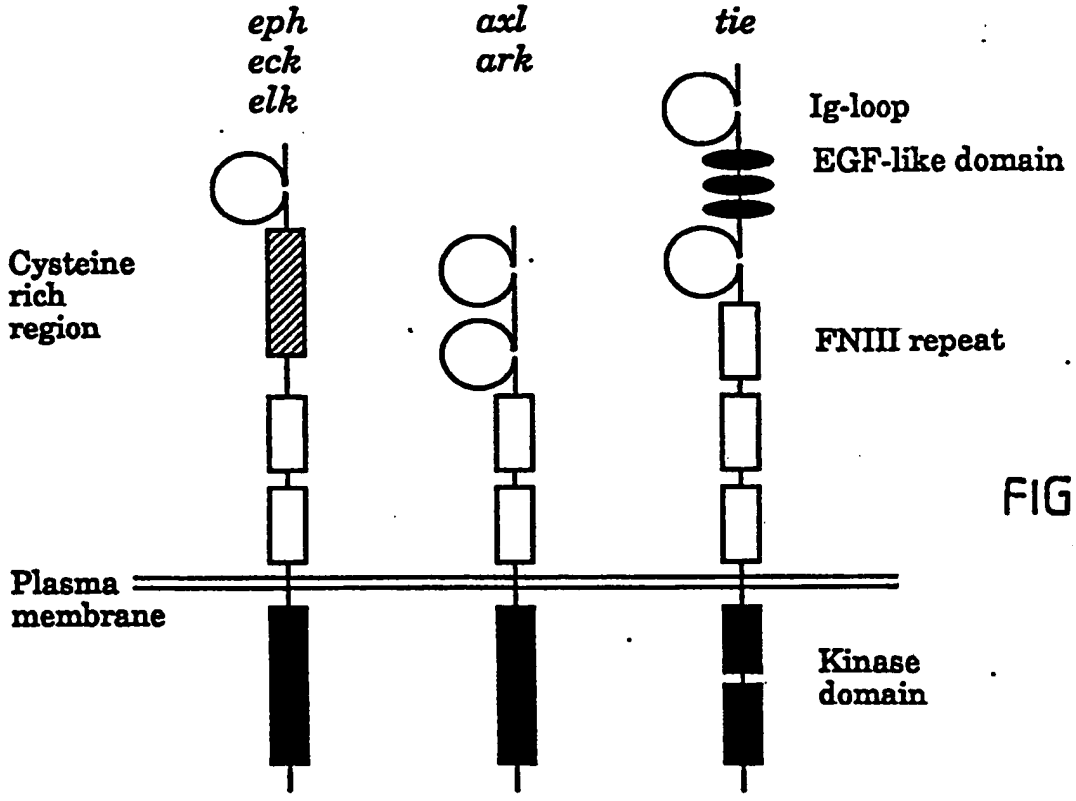

FIG. 9

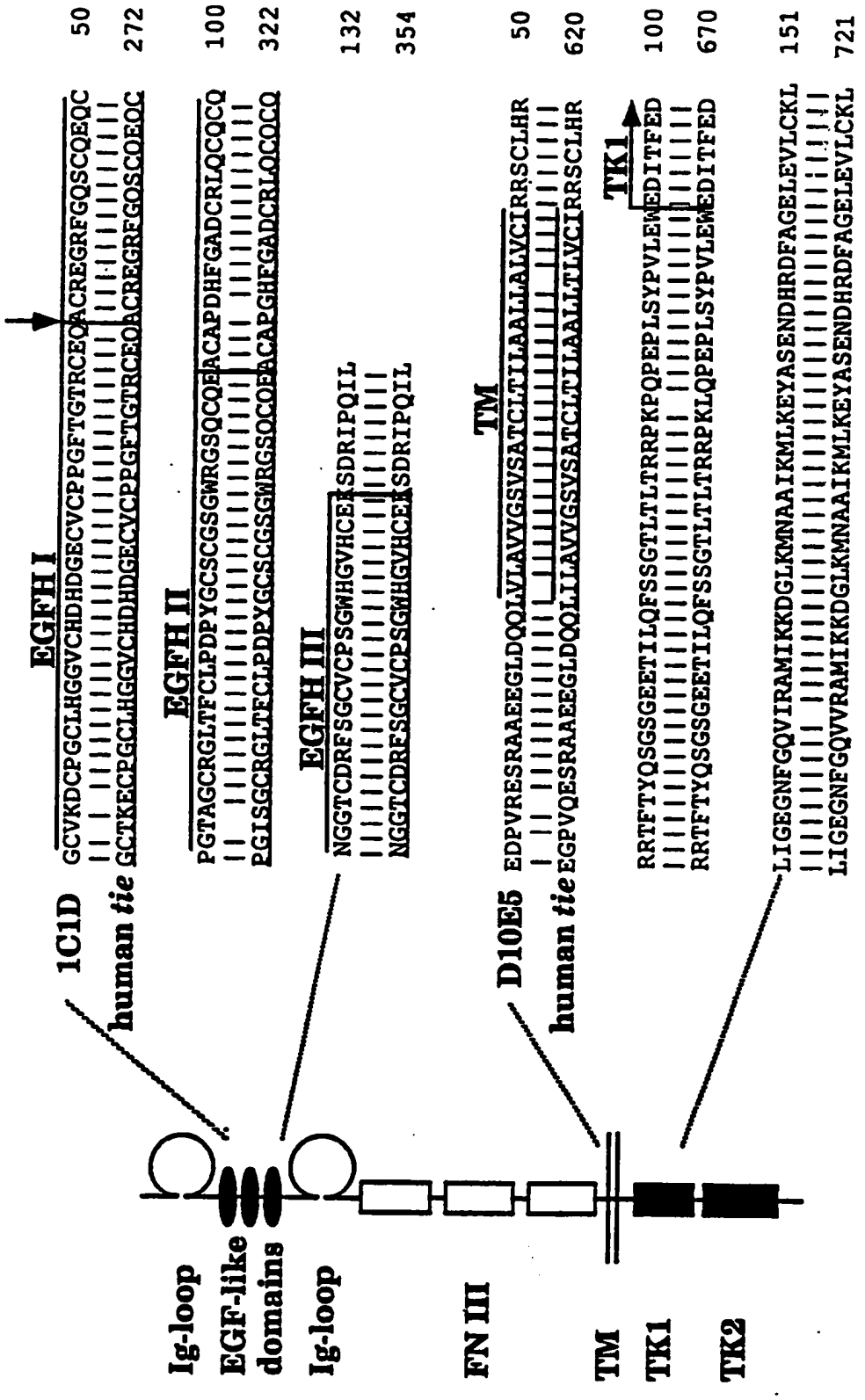
EGFH I
EGFH II
EGFH III
TM
TK1
1C1D
human tie
GCVKDCPGCLHGGVCHDHDGECVCPPGFTGTRCEQACREGRFGQSCQEQC
GCTKECPGCLHGGVCHDHDGECVCPPGFTGTRCEQACREGRFGQSCQEQC
50
272
PGTAGCRGLTFCLPDPYGCSCGSGWRGSQCQEACAPDHFGADCRLQCQCQ
PGISGCRGLTFCLPDPYGCSCGSGWRGSQCQEACAPGHFGADCRLQCQCQ
100
322
NGGTCDRFSGCVCPSGWHGVHCEKSDRIPQIL
NGGTCDRFSGCVCPSGWHGVHCEKSDRIPQIL
132
354
D10E5
human tie
EDPVRESRAAEEGLDQQLVLAVVGSVSATCLTLIAALLIAIVCIRRSCLHR
EGPVQESRAAEEGLDQQLILAVVGSVSATCLTLIAALLTLVCIRRSCLHR
50
620
RRTFTYQSGSGEETILQFSSGTLTLTRRPKPQPEPLSYPVLEWEDITFED
RRTFTYQSGSGEETILQFSSGTLTLTRRPKLQPEPLSYPVLEWEDITFED
100
670
LIGEGNFGQVIRAMIKKDGLKMNAAIKMLKEYASENDHRDFAGELEVLCKL
LIGEGNFGQVVRAMIKKDGLKMNAAIKMLKEYASENDHRDFAGELEVLCKL
151
721
Ig-loop
EGF-like domains
Ig-loop
FN III
TM
TK1
TK2

FIG. 10

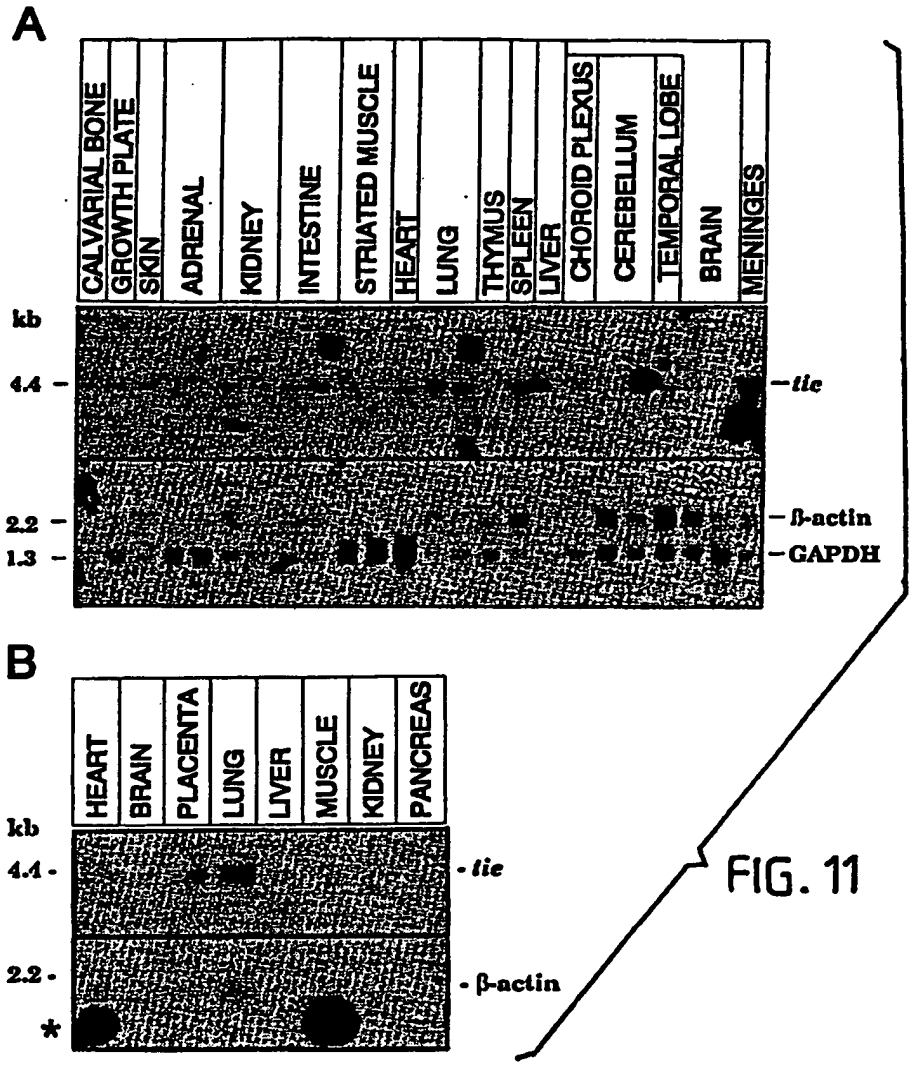
A
CALVARIAL BONE
GROWTH PLATE
SKIN
ADRENAL
KIDNEY
INTESTINE
STRIATED MUSCLE
HEART
LUNG
THYMUS
SPLEEN
LIVER
CHOROID PLEXUS
CEREBELLUM
TEMPORAL LOBE
BRAIN
MENINGES
kb
4.4
2.2
1.3
tie
ß-actin
GAPDH
B
HEART
BRAIN
PLACENTA
LUNG
LIVER
MUSCLE
KIDNEY
PANCREAS
kb
4.4
2.2
*
tie
β-actin

FIG. 11

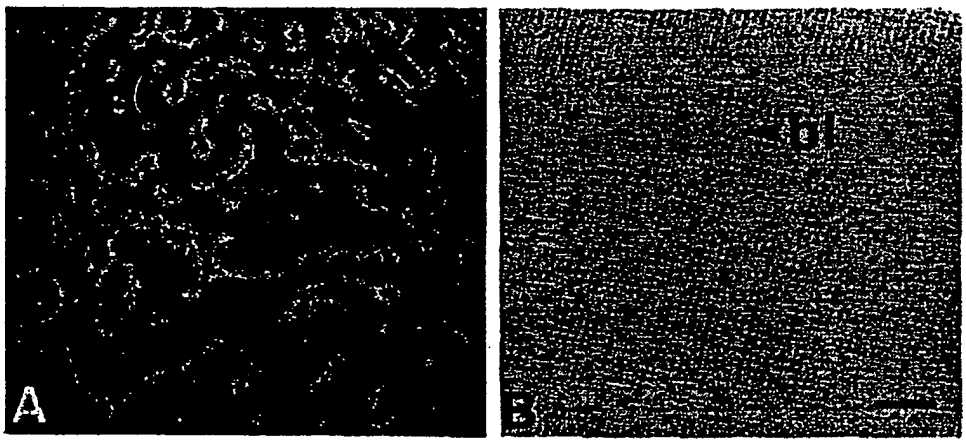
A
B

FIG. 13

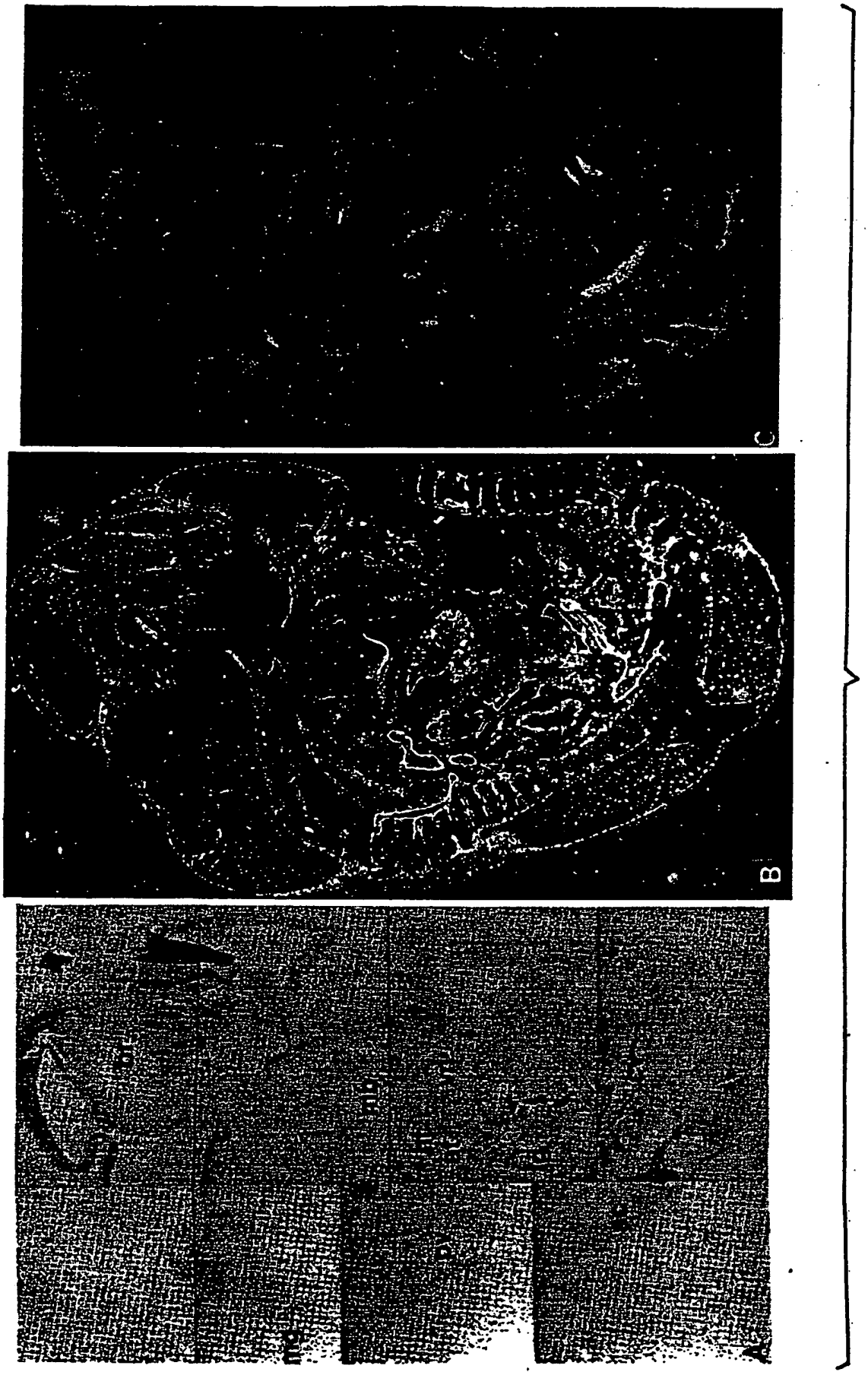

FIG. 12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI 93/00006

A. CLASSIFICATION OF SUBJECT MATTER

IPC5: C07K 13/00, C12N 9/12, C12N 15/54
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC5: C07K, C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, CA, BIOSIS, MEDLINE, EMBL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	EMBL Database entry HSTIEMR, accession no. X60957 02-APR-1992, Partanen J. M. et al:"Human tie mRNA for putative receptor tyrosine kinase" --	1-23
P,X	Dialog Information Services, File 5, BIOSIS, accession no. 9146342, Partanen J. et al: "A novel endothetial cell surface receptor tyrosine kinase with extracellular epidermal growth factor homology domains", Moll cell biol. 12(4), 1992, 1698-1707 --	1-23

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 April 1993	22-04-1993
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Yvonne Siösteen Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI 93/00006

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	Chemical Abstracts, Volume 118, No 11, 15 March 1993 (15.03.93), (Columbus, Ohio, USA), Korhonen, J., "Enhanced expression of the tie receptor tyrosine kinase in endothelial cells during neovascularization", page 518, THE ABSTRACT No 98758f, Blood 1992, 80 (10), 2548-2555, (E)	1-23
A	Chemical Abstracts, Volume 118, No 11, 15 March 1993 (15.03.93), (Columbus, Ohio, USA), Rescigno, J. et al., "A putative receptor tyrosine kinase with unique structural topology", page 346, THE ABSTRACT No 96973y, Oncogene 1991, 6 (10), 1909-1913, (E)	1-23
A	Proc. Natl. Acad. Sci., Volume 87, November 1990, Partanen J. et al., "Putative tyrosine kinases expressed in K-562 human leukemia cells" page 8913 - page 8917	1-23